European Commission

Combined Draft Renewal Assessment Report prepared according to Regulation (EC) N° 1107/2009
and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

GLYPHOSATE

Volume 3 – B.6.6 (AS)

Rapporteur Member State: Assessment Group on Glyphosate (AGG) consisting of FR, HU, NL and SE

Version History

When	What
2021/06	Initial RAR

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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B.6.6.1. Generational studies

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Data point	CA 5.6.1/001
Report author	[redacted] *et al.*
Report year	2007
Report title	Glyphosate technical: Dietary Two Generation Reproduction Study in the Rat
Report No.	2060/0013
Document No.	Not reported
Guidelines followed in study	OECD 416 (2001), JMAFF 2-1-17 (2001), US-EPA OPPTS 870.3800 (1998)
Deviations from current test guideline	None
Previous evaluation	Yes, evaluated and accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Yes

Amendment 1 to final report:

Data point	CA 5.6.1/002
Report author	[redacted]*. et al.*
Report year	2008
Report title	Glyphosate technical: Dietary Two Generation Reproduction Study in the Rat (First amendment to report)
Report No.	2060/0013
Document No.	Not reported
Guidelines followed in study	See final report above
Deviations from current test guideline	See final report above
Previous evaluation	Yes, evaluated and accepted in RAR (2015)
GLP/Officially recognised testing facilities	See final report above
Acceptability/Reliability	Yes

Amendment 2 to final report:

Data point	CA 5.6.1/003
Report author	[redacted]*. et al.*
Report year	2008
Report title	Glyphosate technical: Dietary Two Generation Reproduction Study in the Rat (Second amendment to report)
Report No.	2060/0013
Document No.	Not reported
Guidelines followed in study	See final report above
Deviations from current test guideline	See final report above
Previous evaluation	Yes, evaluated and accepted in RAR (2015)
GLP/Officially recognised testing facilities	See final report above

Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material: Glyphosate technical
Lot/Batch#: H05H016A
Purity/Radiochemical purity: Purity: 95.7% (w/w)
Vehicle: Plain diet
Animals: Species: Rat
Strain: Sprague-Dawley Crl:CD (SD) IGS BR
Age: approximately 8 weeks
Sex: males and females
Weight at dosing: Males: 138-257 g; females: 140-195 g
Acclimation period: At least 14 days
Diet/Food: Rodent PMI 5002 (certified) diet (BCM IPS Limited, UK), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Initially in groups of up to four in polypropylene cages with stainless steel grid floors and tops, suspended over polypropylene trays lined with absorbent paper. During mating animals were house one male: one female. Mated females were housed individually during gestation and lactation in polypropylene cages with solid floors and stainless-steel lids, furnished with softwood flakes.
Environmental conditions:
Temperature: 21 ± 2 °C
Humidity: 55 ± 15 %
Air changes: at least 15/hour
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In a two-generation reproduction study groups of 28 Sprague-Dawley Crl:CD (SD) IGS BR rats per sex of the F0 generation received daily dietary doses of 0, 1500, 5000 and 15000 ppm (equivalent to mean achieved dose levels of 0, 104, 351 and 1063 mg/kg bw/day for males, and 0, 162, 530 and 1634 mg/kg bw/day for females) glyphosate technical in diet. The dose levels were chosen based on results of a previously conducted study (90-day subchronic oral toxicity study in the rat, Report No. 434/016). After 10 weeks of treatment pairing of animals within each dose group was undertaken on a one male: one female basis, to produce the F1 litters. At weaning of offspring from the F0 mating phase, groups of twenty-four male and twenty-four female offspring from each dose group were selected to form the F1 generation. The remaining surviving F0 females and unselected offspring were terminated at Day 21 *post partum*, followed by the termination of all F0 male dose groups. The offspring selected for the F1 generation were dosed for at least 10 weeks and then paired within each dose group to produce the F2 litters. At weaning of the F2 litters all surviving adults and their offspring were killed, followed by the termination of all F1 male dose groups.

Diet preparation and analyses
For preparation of diet mixtures a known amount of the test substance was mixed with a small amount of basal diet at a constant speed for 19 minutes in a Hobart QE200 mixer. This pre-mix was then added to larger amount of basal diet and blended for further 30 minutes in a Hobart H800 mixer.

The stability and homogeneity of the test material in diet were determined. Dietary admixtures were analysed for achieved concentration weekly for the first four weeks of the study and monthly thereafter.

Clinical observations
A check for clinical signs of toxicity, ill-health or behavioural changes was made once daily.

Body weight
Individual body weights were recorded for F0 males on Day 1 (prior to treatment) and at weekly intervals for F0 and F1 males until termination. F0 and F1 females were weighed daily until mating was evident.

Body weights for females showing evidence of mating were recorded on Days 0, 7, 14 and 21 *post coitum*. Females with live litters were weighed on days 1, 4, 7, 14 and 21 *post partum*.

Food consumption and compound intake
During the maturation period, weekly food consumption was recorded for each cage of adults. For females showing evidence of mating, food consumption was recorded for the periods covering days 0-7, 7-14 and 14–21 *post coitum*. For females with live litters, food consumption was recorded for the period covering Days 1-4, 4-7, 7-14, 14-21 *post partum*.

Food conversion efficiency (the ratio of body weight change/dietary intake) was calculated retrospectively for males for both the pre-mating and post-mating phases of the study. For females, food conversion efficiency was only calculated for the pre-mating phases of the study. Due to offspring growth, milk production and weaning, food efficiency could not be accurately calculated for the gestation and lactation phases of the study.

Water consumption
Water intake was observed daily by visual inspection of water bottles for any overt change.

Reproduction parameters
Oestrus cycle
Prior to pairing of females for the F0 and F1 mating phases, a vaginal smear was taken daily for twenty-one days and examined microscopically to determine the stage of oestrous.

Mating
F0 and F1 animals were paired, following subsequent maturation periods, on a 1 male: 1 female basis within each dose group, for a period of up to 21 days. Cage tray-liners were checked each morning for the presence of ejected copulation plugs and each female was examined for the presence of a copulation plug in the vagina. A vaginal smear was prepared for each female and the stage of the oestrous cycle or the presence of sperm was recorded.

The presence of sperm within the vaginal smear and/or vaginal plug in situ was taken as positive evidence of mating and the males were subsequently returned to their original holding cages. Mated females were housed individually during the period of gestation and lactation.

Pregnancy and parturition
Pregnant females were observed at approximately 0830, 1230 and 1630 hours daily, and at approximately 0830 and 1230 hours on weekends and public holidays. In addition, the females were observed around the period of expected parturition. The date of mating, date and time of start and end of parturition and duration of gestation was recorded.

Litter data
The following litter data were recorded:
The number of offspring born, the number of offspring alive recorded daily and reported on Day 1, 4, 7, 14, 21 *post partum*. On Days 1, 4 and 21, the sex of individual offspring was recorded. The clinical condition of offspring were recorded form birth to weaning. Individual offspring and total litter weights were recorded on Day 1, 4, 7, 14 and 21 *post partum*.

Physical and sexual development
All live offspring were observed for the detachment and unfolding of pinna, incisor eruption and eyelid separation and assessed for reflexological response to stimuli by assessing surface righting reflex on Day 1 *post partum* and air righting reflex on Day 17 *post partum*. Pupillary reflex and auditory startle response were performed on Day 21 *post partum*.

All selected F1 offspring were observed for sexual development and the body weight for each individual animal at the time of sexual maturation was recorded. For females the day of appearance of vaginal opening (separation of the labia) was recorded; for males the day of separation of the prepuce from the *glans penis* was recorded. In addition, the ano-genital distance was recorded for all F2 generation offspring on Day 1 *post partum*.

Sacrifice and pathology
All surviving adult females and surviving offspring, except offspring selected to form the F1 generation, as well as surviving males were sacrificed on Day 21 *post partum*.

All adult animals and offspring, including those dying during the study, were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded. For females the uterine implantation sites were counted. In addition, the corpora lutea of all ovaries from pregnant females were counted at necropsy.

The following organs of F0 males and females from each dose group that were sacrificed at the end of the study sampled, weighed and preserved, except for the thyroids, which were weight after fixation: adrenals, brain, left cauda epididymis, epididymides, kidneys, liver, ovaries, prostrate, pituitary, seminal vesicles (with coagulating gland and fluids), spleen, testes, thymus, thyroid glands, and uterus (with cervix and oviducts).

The following organs from one male and one female offspring from the F0 and F1 pairings were weighed: brain, spleen, thymus, and uterus.

The following tissues were preserved from all F0 males and females from each dose group in 10 % buffered formalin, except for the right epididymis, right testis, which were fixed in Bouins fluid and 70 % IMS: adrenals, coagulating gland, right epididymis, ovaries, right testis, pituitary, prostrate, seminal vesicles, uterus (with oviducts) and cervix, vagina and all gross lesions.

A detailed histopathological examination was performed on all sampled tissues from all F0 and F1 control and high-dose animals, and on animals that died or were killed in extremis.

During the histopathological examination there were indications of treatment-related changes in the adrenal glands for the F1 animals. Thus, the microscopic examination was subsequently extended to include similarly prepared sections of adrenals from the F1 animals from the 5000 and 1500 ppm dose groups.

Semen assessment
At necropsy of adult F0 and F1 males at least 200 individual sperms were evaluated for motility, motility characteristics, and morphology. In addition, samples of the testis and cauda epididymis of the control and high dose animals were homogenised and examined for homogenisation resistant spermatids.

Evaluation of the oocyte number
From ten control and ten high dose females of the F1 generation slides of the ovaries were prepared and analysed for visible oocytes. The identified oocytes were classified as small, medium or large follicles.

Statistics
Organ weight (absolute and relative to terminal body weight), weekly body weight gain, litter weights and offspring body weights were assessed for dose response relationships by linear regression analysis, followed by one way analysis of variance (ANOVA) incorporating Levene's test for homogeneity of variance. Where variances were shown to be homogenous, pair wise comparisons were conducted using Dunnett's test. Where Levene's test showed unequal variances the data were analysed using non-parametric methods: Kruskal-Wallis ANOVA and Mann-Whitney 'U' test.

The non-parametric methods were also used to analyse implantation loss, offspring sex ratio and developmental landmarks and reflexological responses.

Probability values (p) are presented as follows:
$p<0.001$ ***
$p<0.01$ **
$p<0.05$ *
$p\geq0.05$ (not significant)

Histopathology data were analysed using the following methods to determine significant differences between control and treatment groups for the individual sexes:

1. Chi-squared analysis for differences in the incidence of lesions occurring with an overall frequency of one or greater.

2. Kruskal-Wallis one-way non-parametric analysis of variance for the comparison of severity grades for the more frequently observed graded conditions.

Probability values (p) were calculated as follows:
$p<0.001$ +++ --- ***
$p<0.01$ ++ -- **
$p<0.05$ + - *
$p<0.1$ (+) (-) (*)
$p\geq0.1$ N.S. (not significant)
(+)-signs indicate positive differences from the control group, and (-)-signs indicate negative differences.
* refer to overall differences between group variation which is non-directional.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS
Stability analyses indicated that the dose preparations at nominal concentrations of 1500, 5000 and 15000 ppm were stable for at least six weeks at ambient temperature.

Analyses for homogeneity at the start of treatment indicated that the dose preparations were homogeneous. Analyses for achieved concentration performed on ten separate occasions demonstrated that the prepared dietary admixture concentrations given to the animals were in the range of 83 to 102 % of the nominal concentration.

TEST COMPOUND INTAKE
The group mean achieved dosages are summarised in the table below.

Table B.6.6.1/01-01: Group mean achieved dose levels

Group	Dietary concentration	Estimated dose level	Mean achieved dose level			
	(ppm)	(mg/kg bw/day)	(mg/kg bw/day)			
			Males	Females		
				Maturation	Gestation	Lactation
Control	0	0	0	0	0	0
Low	1500	75	104	126	108	252
Intermediate	5000	250	351	423	358	808
High	15000	750	1063	1273	1109	2520

MORTALITY
There were no test substance-related mortalities.

Four unscheduled deaths occurred during the study. In the F0 generation one male of the low dose group and one female of the mid dose group was killed for humane reasons on Days 84 and 103, respectively. The male exhibited a mass of about 3 x 4 cm on the lower jaw. The female was in extremis following a suspected prolonged parturition. One high dose female was found dead on day 97 possibly due to complications during parturition.

In the F1 generation one control female was killed on day 99 following severe clinical signs (pallor of the extremities, lethargy, pilo-erection, hunched posture and staining around the ano-genital region); however, the aetiology of the signs was not established.

CLINICAL OBSERVATIONS
No treatment-related clinical signs of toxicity were noted. Clinical signs observed in control and treated animals of the F0 and F1 generation are summarised in the tables below. These signs were considered unrelated to the test substance, since they were either commonly seen in laboratory rats, or caused by physical injury, or occurred in control and treated rats.

Table B.6.6.1/01-02: Observed clinical signs in F0 generation

Clinical sign	Number of rats affected in dose group*							
	Control (0 ppm)		Low (1500 ppm)		Mid (5000ppm)		High (15000 ppm)	
	♂	♀	♂	♀	♂	♀	♂	♀
Abrasion to dorsal region	2/28	0/28	0/28	0/28	0/28	0/28	1/28	0/28
Generalised fur loss	5/28	5/28	3/28	5/28	2/28	6/28	2/28	3/28
Red/brown staining around snout	4/28	0/28	4/28	0/28	1/28	3/28	5/28	0/28
Red/brown staining of fur	1/28	0/28	1/28	0/28	2/28	2/28	1/28	0/28
Red/brown staining around eyes	1/28	0/28	1/28	1/28	0/28	0/28	3/28	0/28
Swollen face (due to overgrowth tooth)	1/28	0/28	0/28	0/28	0/28	0/28	0/28	0/28
Cranial abrasion	0/28	0/28	0/28	0/28	0/28	0/28	2/28	0/28
Red stained urine	0/28	0/28	0/28	0/28	0/28	0/28	1/28	0/28
Facial scab formation	1/28	0/28	0/28	0/28	1/28	0/28	0/28	0/28
Scab formation	1/28	0/28	1/28	0/28	1/28	0/28	0/28	0/28
Large mass under lower jar	0/28	0/28	1/28	0/28	0/28	0/28	0/28	0/28
Mass on dorsal region	0/28	0/28	0/28	0/28	1/28	0/28	0/28	0/28
Scab formation around right eye	0/28	0/28	0/28	0/28	1/28	0/28	0/28	0/28
Physical injury to tail apex	0/28	0/28	0/28	1/28	0/28	0/28	0/28	0/28
Stained fur on head	0/28	0/28	0/28	0/28	0/28	0/28	0/28	1/28
Red swollen ears	0/28	0/28	0/28	0/28	0/28	1/28	0/28	1/28
Blood seen without evidence of offspring born	0/28	1/28	0/28	0/28	028	0/28	0/28	0/28
Blood around vagina (suspected prolonged parturition, killed in extremis)	0/28	0/28	0/28	0/28	0/28	1/28	0/28	0/28
Pilo-erection	0/28	0/28	0/28	0/28	0/28	0/28	0/28	1/28
Exophthalmia	0/28	0/28	0/28	0/28	0/28	0/28	0/28	1/28

* x/y: number affected / total number of animals in group

Table B.6.6.1/01-03: Observed clinical signs in F1 generation

Clinical sign	Number of rats affected in dose group*							
	Control (0 ppm)		Low (1500 ppm)		Mid (5000ppm)		High (15000 ppm)	
	♂	♀	♂	♀	♂	♀	♂	♀
Generalised fur loss	3/28	4/28	0/28	2/28	0/28	6/28	0/28	4/28
Red/brown staining around eyes	2/28	1/28	0/28	0/28	1/28	0/28	0/28	0/28
Red/brown staining of fur	0/28	1/28	2/28	0/28	0/28	2/28	1/28	1/28
Red/brown staining around snout	1/28	7/28	1/28	0/28	4/28	7/28	1/28	4/28
Scabbing and fur loss around eye	0/28	0/28	0/28	0/28	0/28	0/28	1/28	0/28
Protruding sternum	0/28	2/28	0/28	3/28	0/28	3/28	0/28	0/28
Lethargy	0/28	1/28	0/28	0/28	0/28	0/28	0/28	0/28
Hunched posture	0/28	1/28	0/28	0/28	0/28	0/28	0/28	0/28
Staining around ano-genital region	1/28	0/28	0/28	0/28	0/28	0/28	0/28	0/28
Pallor of extremities	1/28	0/28	0/28	0/28	0/28	0/28	0/28	0/28

* x/y: number affected / total number of animals in group

BODY WEIGHT

No adverse effect of body weight change was evident for treated animals in comparison to controls throughout the treatment period for both the F0 and F1 generations. During the final week of lactation, both the F0 and F1 generations showed statistically significant less body weight loss in comparison to controls ($p < 0.001$ and $p< 0.01$ respectively). There was no difference in the litter size or offspring weights at this dietary concentration, therefore this superior performance in comparison to control was not considered an adverse effect.

Table B.6.6.1/01-04: Body weight changes during lactation (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight Change (g) at Day			
			4	7	14	21
			F0 Generation			
0 (Control)	26	mean	15	22	0	-23
		sd	14	9	15	10
1500	27	mean	16	16	3	-26
		sd	9	9	13	13
5000	26/27•	mean	16	18	1	-23
		sd	14	13	11	11
15000	26	mean	18	18	1	-8***
		sd	11	12	14	14
			F1 Generation			
0 (Control)	22	mean	14	9	9	-16
		sd	11	13	14	13
1500	23	mean	14	16	3	-21
		sd	7	11	9	17
5000	24	mean	17	10	5	-17
		sd	12	10	13	13
15000	23	mean	16	11	10	-4**
		sd	9	9	12	13

S standard deviation
** significantly different from control group $p < 0.01$
*** significantly different from control group $p < 0.001$
•=n-26 Day 1, n=27 Day 4 onwards

FOOD CONSUMPTION

Males:

No adverse effect on dietary intake or food conversion efficiency was evident for either the F0 or F1 males during the pre-mating or post-mating phases of the study.

Females:

There were no adverse effects on dietary intake for treated females from both the F0 and F1 generations, when compared to concurrent control. No adverse effects of dietary intake or food conversion efficiency were evident for F0 females during the pre-mating phases of the study. Dietary intake during the gestation or lactation phases for treated animals was also comparable to controls. Food conversion efficiencies were not calculated for the gestation and lactation phases of the study. Statistical analysis of the data did not reveal any adverse intergroup differences.

WATER CONSUMPTION

Daily visual inspection of water bottles showed no overt intergroup differences in water intake for treated males and females from the F0 or F1 generations, when compared to their concurrent controls.

REPRODUCTIVE PARAMETERS

Oestrus cycle

There were no toxicologically-significant effects on female oestrous cycles. The higher number of irregular cycles for F1 generation females treated with 15000 and 5000 ppm in comparison to controls, were not reflected in the pre-coital intervals suggesting there was no difference in mating performance. These intergroup differences were considered to have arisen incidentally and were of no toxicological significance according to study author.

Mating Performance, Fertility and Gestation

There were no treatment-related effects on mating performance, fertility and gestation length for both F0 and F1 generation animals.

Table B.6.6.1/01-05: Mating, fertility and gestation parameters (F0)

	Dose (ppm)		**0**		**1500**		**5000**		**15000**	
Oestrous cycle-prior to mating (F0)	Regular 4 or 5d cycles		24/28 (86%)		26/28 (93%)		20/28 /71%)		24/28 (86%)	
	Irregular cycle (a)		3/28 (11%)		1/28 (3.5%)		3/28 (11%)		2/28 (7%)	
	Acyclic (b)		1/28 (3.5%)		1/28 (3.5%)		3/28 (11%)		1/28 (3.5%)	
	Pseudo-pregnancy		0/28		0/28		2/28		1/28	
Pre-coital interval (F0)	1		9/28 (32%)		2/28 (7%)		8/28 (29%)		8/28 (29%)	
	2-4		16/28 (57%)		24/28 (86%)		17/28 (61%)		16/28 (57%)	
	>4		3/28		2/28+		3/28		4/28	
Mating performance (F0)			M	F	M	F	M	F	M	F
	#Paired		28	28	28	28	28	28	28	28
	#Successful mating		28/28 (100%)		28/28 (100%)		28/28 (100%)		28/28 (100%)	
	#Pregnant			27!		27		28		28
	#Females with live offspring			26		27		28		27++
Gestation length	Gestation length (days)	22		13 (50%)		11 (41%)		9 (32%)		8 (30%)
		22-23		12 (46%)		16 (59%)		19 (68%)		18 (67%)
		>23		1 (4%)		0 (0%)		0 (0%)		1 (3%)
Number of litters			26[1]		27		27$		26[1]	
Pre-implantation loss (%) ±SD			6.5±6.6		8.0±12.0		6.7±5.5		7.4±7.0	
Post-implantation loss (%) ±SD			5.1±6.5		4.9±6.3		4.2±4.9		5.1±8.3	

(a) at least one cycle of two, three, or six to ten days; (b): extended oestrus or di-oestrus
! One female with implantation sites was not observed to give birth to a litter.
+ Male partner killed after first week of pairing and replaced.
++ Includes one female with total litter loss.
1 25 litters used in calculation of pre-implantation loss
$ 26 litters used to calculate pre and post implantation loss

Table B.6.6.1/01-06: Mating, fertility and gestation parameters (F1)

	Dose (ppm)		**0**		**1500**		**5000**		**15000**	
Oestrous cycle-prior to mating (F0)	Regular 4 or 5d cycles		21/24 (88%)		21/24 (88%)		17/24 (71%)		15/24 (62.5%)	
	Irregular cycle (a)		2/24 (8%)		2/24 (8%)		5/24 (21%)		6/24 (25%)	
	Acyclic (b)		1/24 (4%)		1/24 (4%)		2/24 (8%)		3/24 (12.5%)	
	Pseudo-pregnancy		0/24		0/24		0/24		1/24	
Pre-coital interval (F0)	1		5/24 (21%)		7/24 (29%)		2/24 (8%)		6/24 (25%)	
	2-4		19/24 (79%)		14/24 (58%)		17/24 (71%)		17/24 (71%)	
	>4		0/24 (0%)		3/24 (13%)		4/24 (17%)		1/24 (4%)	
Mating performance (F0)			M	F	M	F	M	F	M	F
	#Paired		24	24	24	24	24	24	24	24
	#Successful mating		24/24 (100%)		24/24 (100%)		24/24 (100%)		24/24 (100%)	
	#Pregnant			22		23		24!		23
Gestation length	Gestation length (days)	22		10 (45%)		11 (48%)		8 (33%)		14 (61%)
		>22		12		12		15		9
				55%		(52%)		(62.5%)		(39%)
Number of litters (pre and post impl. calculated			22 (21)		23 (22)		24 (23)		23 (21)	

for)				
Pre-implantation loss (%) ±SD	13.5±14.4	9.3±8.0	13.1±12.1	8.6±5.7
Post-implantation loss (%) ±SD	12.3±15.6	5.4±7.1	6.0±8.7	7.0±6.4

! One female showed no signs of mating but went on to give birth to live offspring
(a) at least one cycle of two, three, or six to ten days; (b): extended oestrus or di-oestrus

LITTER DATA

Size and Viability

No overt differences in litter size and viability were detected. The mean numbers of corpora lutea and subsequent number of implantations did not indicate any adverse effect of dietary exposure and pre and post implantation loss for treated animals were essentially similar to controls. There were no toxicologically significant differences in sex ratio for both F0 - F1 and F1 - F2 litters.

Table B.6.6.1/01-07: Litter size-F0-F1 generation (group mean values)

	Dose (ppm)	**0**	**1500**	**5000**	**15000**
#Litters		26 (25)•	27	27	26 (25)•
#Corpora lutea		17.4±1.9	15.9±2.3	17.6±2.1	17.2±2.1
#Implantation sites		16.3±2.0	14.8±3.1	16.3±1.9	15.9±2.2
#Offspring born		15.4±1.7	14.1±3.1	15.7±2.2	15.1±2.2
#Alive	Age (day)				
	1	15.2±1.6	14.0±3.1	15.4±2.1	14.7±2.2
	4	15.2±1.6	13.8±3.1	15.4±2.1	14.5±2.1
	7	15.1±1.7	13.8±3.1	15.2±2.0	14.3±2.0
	14	14.8±1.7	13.5±2.9	13.9±2.4	13.8±2.9
	21	14.8±1.8	13.4±2.9	13.8±2.5	13.8±2.9
Total litter weight (g)	Day (post-partum)				
	1	107.3±8.2	99.2±20.9	109.4±15.2	103.4±12.3
	4	156.1±10.9	145.4±29.9	156.5±20.0	148.2±15.3
	7	225.1±16.7	213.2±40.7	222.2±25.6	213.6±23.4
	14	407.5±26.0	388.1±70.9	395.8±49.7	380.7±70.4
	21	660.8±46.4	634.8±115.9	634.3±88.4	606.7±115.1

Sd= standard deviation
•=n=25 for total number of corpora lutea

Table B.6.6.1/01-08: Survival indices – F0-F1 generation (group mean litter values)

	Dose (ppm)	**0**	**1500**	**5000**	**15000**
#Litters examined		26 •	27	27‣	26 •
	Age (day)				
Live birth index	1	98.8±2.9	99.2±3.0	98.2±3.3	97.2±4.3
Viability index	1	99.5±2.5	98.9±2.9	100±0.0	98.7±2.7
	4	99.5±1.9	99.8±1.1	99.0±3.3	99.1±2.9
	7	98.5±3.3	98.1±4.2	92.0±13.3	96.5±15.4
	14	99.7±1.4	99.8±1.3	99.1±2.7	100.0±0.0
	21	97.2±5.0	96.6±5.4	90.5±14.8	94.9±16.1
Total litter weight (g)	Day (post-partum)				
	1	99.1±22.4	102.8±14.9	106.0±18.0	105.0±11.7
	4	144.5±31.2	154.2±20.3	154.1±23.1	152.9±17.6
	7	206.7±44.4	224.8±28.6	221.3±31.9	218.8±21.8
	14	361.0±94.9	406.4±43.1	379.2±90.3	378.9±61.0
	21	576.8±154.0	661.6±71.7	605.9±149.5	603.3±97.3

•=25 litter used in calculation of pre-implantation loss
‣=26 litters used to calculated pre and post implantation loss
sd=standard deviation

Table B.6.6.1/01-09: Litter size – F1-F2 generation (group mean values)

	Dose (ppm)	0	1500	5000	15000
#Litters		22(21)•	23(22)‣	24(23)†	23(21)•
#Corpora lutea		18.0±1.9	17.0±1.7	18.2±2.5	17.5±1.7
#Implantation sites		15.5±2.6	15.4±2.1	15.7±2.4	16.0±1.7
#Offspring born		13.8±3.5	14.7±2.3	14.9±2.8	15.1±1.8
#Alive	Age (day)				
	1	13.7±3.4	14.5±2.3	14.5±3.2	14.9±1.8
	4	13.7±3.4	14.3±2.2	14.5±3.1	14.7±1.9
	7	13.5±3.4	14.3±2.2	14.2±2.9	14.6±2.0
	14	12.4±3.8	13.9±2.1	12.9±3.8	13.6±2.7
	21	12.2±4.0	13.8±2.0	12.8±3.9	13.6±2.7

•=n=21 for total number corpora lutea and total number implantation sites
‣=n=22 for total number implantation sites
†=n=23 for total number corpora lutea and total number implantation sites
sd=standard deviation

Table B.6.6.1/01-10: Survival indices- F1-F2 generation

	Dose (ppm)	0	1500	5000	15000
#Litters examined		22/21•	23/22‣	24/23†	23/21‡
	Age (day)				
Live birth index	1	99.1±2.3	98.3±3.5	97.1±8.7	98.6±4.1
Viability index	1	100.0±0.0	99.2±2.1	99.6±1.9	99.1±2.5
	4	99.1±2.4	99.3±2.2	98.3±3.9	99.1±3.1
	7	92.0±16.2	97.6±6.4	91.6±19.8	93.5±16.4
	14	97.1±7.7	99.7±1.4	98.4±5.4	100.0±0.0
	21	89.5±19.1	96.0±8.2	89.0±21.1	92.1±17.0

Values expressed in group mean ±SD
•=n=21 for pre-implantation loss and post-implantation loss
‣=n=22 for pre-implantation loss and post-implantation loss
†=n=23 for pre-implantation loss and post-implantation loss
‡=n=21 for pre-implantation loss and post-implantation loss
sd=standard deviation

Offspring growth and development

No adverse effects on mean offspring bodyweights, bodyweight change or development were detected for male and female offspring in comparison to their controls.

There were no significant inter-group differences in offspring maturation, as assessed by the onset and completion of pinna unfolding, incisor eruption and eye opening for offspring from either the F0-F1 and F1-F2 generations.

F_0 - F_1 Generation

Dietary Concentration (ppm)	Number of Litters		Day *post partum*: Pinna Unfolding Onset	Pinna Unfolding Completion	Incisor Eruption Onset	Incisor Eruption Completion	Eye Opening Onset	Eye Opening Completion
0 (Control)	26	mean	2.4	3.1	9.5	12.4	17.4	19.6
		sd	0.7	0.6	0.6	1.2	0.5	0.8
1500	27	mean	2.3	3.0	9.6	12.3	17.3	19.2
		sd	0.7	0.7	0.8	2.0	0.6	1.0
5000	27	mean	2.5	3.3	9.8	12.4	17.3	19.5
		sd	0.6	0.9	0.7	1.0	0.7	1.1
15000	26	mean	2.3	3.3	9.6	12.3	17.4	19.5
		sd	0.7	0.8	0.9	1.2	0.6	1.1

F_1 - F_2 Generation

Dietary Concentration (ppm)	Number of Litters		Day *post partum*: Pinna Unfolding Onset	Pinna Unfolding Completion	Incisor eruption Onset	Incisor eruption Completion	Eye opening Onset	Eye opening Completion
0 (Control)	22	mean	2.9	4.5	9.8	12.9	17.6	20.1
		sd	0.6	1.0	1.1	1.2	0.8	0.8
1500	23	mean	3.0	4.3	10.1	13.3	17.3	20.3
		sd	0.6	0.8	0.9	1.1	1.1	0.6
5000	24	mean	2.8	4.4	10.1	13.0	17.8	19.9
		sd	0.7	0.8	1.3	1.7	1.0	1.1
15000	23	mean	3.1	4.7	9.8	12.7	17.9	20.5
		sd	0.7	0.8	1.2	1.1	1.6	0.8

The reflexological assessments (percentage successful at surface righting, mid-air righting and pupil and startle reflex) did not indicate any toxicologically significant effects of dietary exposure at 1500, 5000 or 15000 ppm. Significantly lower numbers of F1-F2 offspring from all treated groups passed the mid-air righting assessment in comparison to all control offspring, however, there was no dose-related trend associated with the lower values and a similar effect was not observed in the F0-F1 offspring. In isolation, the statistically significant differences in this parameter were considered to have arisen from normal biological variation and were considered not to represent a toxic effect of the test material according to study author.

Table B.6.6.1/01-11: Group mean (%) litter values with successful reflexological response– F0-F1 and F1-F2

	Dose (ppm)	**0**	**1500**	**5000**	**15000**
F0-F1					
Number of litters		26	27	27	26
Surface righting		97.1	95.0	93.0	91.3
Mid-air righting		98.1	99.8	99.5	99.8
Pupil reflex		100.0	100.0	100.0	100.0
Startle reflex		100.0	100.0	100.0	100.0
F1-F2					
Number of litters		22	23	24	23
Surface righting		87.0	90.5	81.3	81.9
Mid-air righting		100.0	98.6*	94.2**	97.1*
Pupil reflex		100.0	100.0	100.0	100.0
Startle reflex		100.0	100.0	100.0	100.0

*significantly different from control group $p<0.05$
** significantly different from control group $p<0.01$

Clinical signs of offspring
No clinically observable signs of toxicity were observed for offspring from treated animals.

Sexual maturation of the F1 generation
Selected F1 generation males treated with the 15000 ppm achieved sexual maturation at a later age, hence a higher mean bodyweight, for the completion of sexual maturation ($p<0.01$) in comparison to controls. No such effects were evident for females and there were no differences in mating performance, sperm changes and histopathological examinations did not reveal any changes in the testis or epididymis.

Table B.6.6.1/01-12: Balano-preputial separation of F1 males

Dietary concentration (ppm)	No. of animals		Age (days) at completion	Body weight (g) at attainment
0 (Control)	24	mean SD	43.0 2.3	210 23
1500	24	mean SD	43.3 1.6	216 22
5000	24	mean SD	43.5 2.3	219 22
15000	24	mean SD	45.9** 3.1	230** 28

SD standard deviation
** significantly different from control group $p<0.01$

Table B.6.6.1/01-13: separation of the labia of F1 females

Dietary concentration (ppm)	No. of animals		Age (days) at completion	Body weight (g) at attainment
0 (Control)	24	mean SD	34.0 1.8	116 13
1500	24	mean SD	33.5 2.1	115 12
5000	24	mean SD	33.6 2.3	118 14
15000	24	mean SD	34.1 1.9	119 14

SD=standard deviation

Ano-genital distance of the F3 generation
There were no significant inter-group differences in ano-genital distance measured for males and females from the F1 treated animals in comparison to the controls.

Table B.6.6.1/01-14: Ano-genital distance of F2 generation (group mean litter values)

Dietary concentration (ppm)	No. of animals		Ano-Genital Distance (mm) on Day 1 *post partum*	
			Males	Females
0 (Control)	22	mean SD	4.62 0.50	2.50 0.47
1500	23	mean SD	4.34 0.39	2.38 0.41
5000	24	mean SD	4.55 0.52	2.73 0.51
15000	23	mean SD	4.70 0.49	2.69 0.41

SD=standard deviation

PATHOLOGY
Adult and offspring necropsy
There were no toxicologically significant macroscopic abnormalities detected in the F0 and F1 animals, nor in the offspring.

Organ weights adults

F0 females treated with 15000 ppm displayed statistically significant increases in liver weights, both absolute and relative to terminal body weight ($p < 0.001$). An increase in liver weights was also noted for F1 females treated with 15000 ppm (absolute: $p < 0.05$, relative: $p < 0.01$). Furthermore, F0 females treated with 15000 ppm displayed an increase in kidney weights, both absolute ($p < 0.001$) and relative to terminal body weight ($p < 0.01$).

No statistically significant changes in liver and kidney were detected for males treated with 15000 ppm from either generation.

F0 males treated with 1500 and 5000 ppm showed statistically significant reductions in relative thyroid weights. No such effects were detected in the 15000 ppm males, therefore, in the absence of a convincing dose-related response, these intergroup differences were considered to be unrelated to treatment. F0 females treated with 5000 ppm showed statistically significant increases in absolute kidney weights and an increase in absolute thyroid weights (22%). However, these increases were not reflected in the relative weights and were considered to be unrelated to treatment according to study author. Increased absolute thyroid weight was also observed in F0 females at the highest dose level (15000 ppm) (19% but was not statistically significant).

F1 males treated with 15000 ppm showed a statistically significant reduction in absolute brain and spleen weights. These reductions were not reflected in the relative weights and in isolation, were considered to have arisen coincidentally.

There were no differences in uterus weights for treated females from either generation when compared to control.

Table B.6.6.1/01-15: Liver, kidney and thyroid weights (relative and absolute) of females (group mean values)

Dietary concentration (ppm)	No. of animals		Organ weight (g)					
			Liver		Kidney		Thyroid	
			Absolute	Relative	Absolute	Relative	Absolute	Relative
			F0 Generation					
0 (Control)	26	mean	15.0328	4.3103	2.4315	0.6977	0.0475	0.0137
		sd	1.0493	0.2864	0.1706	0.0548	0.0087	0.0023
1500	27	mean	15.1465	4.3027	2.5395	0.7233	0.0504	0.0143
		sd	1.4948	0.3435	0.1602	0.0560	0.0136	0.0036
5000	27	mean	15.8791	4.3570	2.5654* (6%)	0.7062	0.0579* (22%)	0.0159
		sd	1.7649	0.2810	0.2361	0.0592	0.0099	0.0026
15000	26	mean	16.9704*** (13%)	4.6806*** (8%)	2.7096*** (11%)	0.7490** (7%)	0.0563 (19%)	0.0156
		sd	1.7620	0.2977	0.2203	0.0521	0.0085	0.0021
			F1 Generation					
0 (Control)	22	mean	16.4887	4.5970	2.6792	0.7483	0.1024	0.0288
		sd	2.0275	0.4038	0.4137	0.1070	0.0212	0.0064
1500	23	mean	16.3848	4.6047	2.5777	0.7257	0.0948	0.0267
		sd	1.7744	0.2858	0.2776	0.0647	0.0192	0.0051
5000	24	mean	17.2591	4.6543	2.8124	0.7585	0.1063	0.0289
		sd	2.0969	0.3628	0.5326	0.1229	0.0164	0.0055
15000	23	mean	18.0724* (10%)	4.9591** (8%)	2.7660	0.7578	0.0937	0.0258
		sd	1.2434	0.3130	0.2616	0.0517	0.0120	0.0039

sd standard deviation
* significantly different from control group $p < 0.05$
** significantly different from control group $p < 0.01$
*** significantly different from control group $p < 0.001$

Table B.6.6.1/01-16: relative organ weights for F0 males -selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)							
				Adrenals	Brain	Epididymides Left	Epididymides Right	Kidneys	Left Cauda	Liver	Pituitary
0 (Control)	28	mean	596	0.0098	0.3668	0.1283	0.1346	0.6694	0.0568	3.1481	0.0020
		sd	60	0.0027	0.0313	0.0214	0.0168	0.0635	0.0110	0.1846	0.0007
1500	27	mean	614	0.0090	0.3505	0.1253	0.1290	0.6514	0.0575	3.0595	0.0021
		sd	59	0.0021	0.0339	0.0180	0.0158	0.0556	0.0098	0.2771	0.0003
5000	28	mean	617	0.0096	0.3484	0.1307	0.1315	0.6603	0.0602	3.0996	0.0019
		sd	50	0.0028	0.0335	0.0176	0.0151	0.0696	0.0114	0.1918	0.0004
15000	28	mean	591	0.0104	0.3657	0.1296	0.1346	0.6961	0.0605	3.2645	0.0023
		sd	73	0.0025	0.0392	0.0144	0.0188	0.0736	0.0092	0.2908	0.0005

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)						
				Prostate	Seminal Vesicles	Spleen	Testes Left	Testes Right	Thymus	Thyroid
0 (Control)	28	mean	596	0.1219	0.4925	0.1501	0.3085	0.3145	0.0672	0.0250
		sd	60	0.0302	0.0948	0.0252	0.0322	0.0344	0.0182	0.0101
1500	27	mean	614	0.1131	0.4712	0.1423	0.2973	0.2992	0.0694	**0.0196
		sd	59	0.0308	0.0826	0.0188	0.0338	0.0333	0.0173	0.0069
5000	28	mean	617	0.1156	0.4954	0.1460	0.2983	0.3040	0.0692	*0.0189
		sd	50	0.0411	0.0958	0.0276	0.0293	0.0286	0.0142	0.0044
15000	28	mean	591	0.1214	0.5170	0.1563	0.3178	0.3126	0.0700	0.0206
		sd	73	0.0296	0.1021	0.0810	0.0402	0.0454	0.0162	0.0045

sd=standard deviation
*=significantly different from control group p<0.05
**= significantly different from control group p<0.01

Table B.6.6.1/01-17: absolute organ weights for F1 males-selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g)							
				Adrenals	Brain	Epididymides Left	Epididymides Right	Kidneys	Left Cauda	Liver	Pituitary
0 (Control)	24	mean	599	0.0896	2.1963	0.7363	0.7594	4.0604	0.3122	19.7293	0.0148
		sd	51	0.1051	0.1172	0.0764	0.0847	0.4264	0.0480	2.1994	0.0045
1500	24	mean	596	0.0607	2.1255	0.8273	0.8585	4.0099	0.4029	19.5341	0.0128
		sd	66	0.0171	0.1023	0.1011	0.1084	0.3983	0.1239	2.6825	0.0042
5000	24	mean	611	0.0600	2.1254	0.8912	0.8412	4.2958	0.3567	20.1228	0.0150
		sd	61	0.0161	0.1253	0.2685	0.0894	0.6573	0.0661	2.2770	0.0040
15000	24	mean	580	0.0525	**2.0975	0.7205	0.8001	4.1505	0.3172	19.3033	0.0175
		sd	45	0.0140	0.0995	0.1422	0.0985	0.3138	0.0331	2.0957	0.0231

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g)						
				Prostate	Seminal Vesicles	Spleen	Testes Left	Testes Right	Thymus	Thyroid
0 (Control)	24	mean	599	0.7789	3.0146	0.8983	1.7672	1.7750	0.4314	0.1277
		sd	51	0.2084	0.4849	0.1428	0.2843	0.2622	0.1038	0.0336
1500	24	mean	596	0.6900	3.0679	0.8890	1.8950	1.8998	0.4878	0.1286
		sd	66	0.1480	0.4526	0.1222	0.1711	0.1767	0.1653	0.0195
5000	24	mean	611	0.7865	2.9590	0.9413	1.9209	1.9091	0.4738	0.1239
		sd	61	0.1679	0.5543	0.1915	0.1683	0.1290	0.0998	0.0255
15000	24	mean	580	0.6826	3.1101	*0.7852	1.8330	1.8717	0.4100	0.1270
		sd	45	0.1465	0.4335	0.1842	0.1477	0.1533	0.1127	0.0225

sd=standard deviation

*=significantly different from control group p<0.05
**=significantly different from control group p<0.01

Table B.6.6.1/01-18: relative organ weights for F1 males- selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)							
				Adrenals	Brain	Epididymides Left	Epididymides Right	Kidneys	Left Cauda	Liver	Pituitary
0 (Control)	24	mean	599	0.0153	0.3687	0.1240	0.1278	0.6824	0.0528	3.2962	0.0025
		sd	51	0.0191	0.0269	0.0178	0.0182	0.0875	0.0104	0.2172	0.0007
1500	24	mean	596	0.0102	0.3601	0.1397	0.1450	0.6748	0.0683	3.2699	0.0021
		sd	66	0.0027	0.0383	0.0188	0.0201	0.0481	0.0229	0.1797	0.0006
5000	24	mean	611	0.0099	0.3512	0.1468	0.1389	0.7080	0.0590	3.2955	0.0025
		sd	61	0.0026	0.0389	0.0445	0.0188	0.1202	0.0127	0.1898	0.0007
15000	24	mean	580	0.0092	0.3632	0.1253	0.1386	0.7177	0.0550	3.3295	0.0029
		sd	45	0.0026	0.0225	0.0259	0.0184	0.0499	0.0071	0.2438	0.0036

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)						
				Prostate	Seminal Vesicles	Spleen	Testes Left	Testes Right	Thymus	Thyroid
0 (Control)	24	mean	599	0.1303	0.5091	0.1506	0.2978	0.2991	0.0725	0.0214
		sd	51	0.0336	0.1034	0.0243	0.0549	0.0521	0.0185	0.0056
1500	24	mean	596	0.1162	0.5168	0.1491	0.3204	0.3213	0.0806	0.0216
		sd	66	0.0248	0.0712	0.0114	0.0376	0.0392	0.0229	0.0028
5000	24	mean	611	0.1297	0.4889	0.1544	0.3172	0.3156	0.0777	0.0205
		sd	61	0.0323	0.1050	0.0288	0.0378	0.0373	0.0151	0.0047
15000	24	mean	580	0.1190	0.5397	0.1351	0.3182	0.3248	0.0705	0.0220
		sd	45	0.0296	0.0853	0.0304	0.0365	0.0364	0.0176	0.0042

sd=standard deviation

Table B.6.6.1/01-19: absolute organ weights for F0 females- selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g)				
				Adrenals	Brain	Kidneys	Liver	Ovaries
0 (Control)	26	mean	349	0.0821	1.9629	2.4315	15.0328	0.1292
		sd	20	0.0139	0.0930	0.1706	1.0493	0.0263
1500	27	mean	352	0.0806	1.9566	2.5395	15.1465	0.1328
		sd	27	0.0154	0.0807	0.1602	1.4948	0.0283
5000	27	mean	364	0.0800	1.9614	*2.5654	15.8791	0.1331
		sd	26	0.0132	0.0800	0.2361	1.7649	0.0212
15000	26	mean	362	0.0827	1.9745	***2.7096	***16.9704	0.1231
		sd	28	0.0107	0.0689	0.2203	1.7620	0.0213

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g)				
				Pituitary	Spleen	Thymus	Thyroid	Uterus
0 (Control)	26	mean	349	0.0150	0.5991	0.1622	0.0475	0.9071
		sd	20	0.0030	0.0777	0.0539	0.0087	0.4754
1500	27	mean	352	0.0139	0.6063	0.1789	0.0504	0.8579
		sd	27	0.0035	0.0961	0.0679	0.0136	0.4507
5000	27	mean	364	0.0151	0.6037	0.1810	*0.0579	0.9378
		sd	26	0.0034	0.1028	0.0451	0.0099	0.4743
15000	26	mean	362	0.0151	0.5824	0.1585	0.0563	0.7508
		sd	28	0.0028	0.0755	0.0544	0.0085	0.5229

sd=standard deviation
*=significantly different from control group p<0.05
***=significantly different from control group p<0.001

Table B.6.6.1/01-20: absolute organ weights for F1 females- selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g) Adrenals	Brain	Kidneys	Liver	Ovaries
0 (Control)	22	mean	358	0.0903	1.9433	2.6792	16.4887	0.1277
		sd	24	0.0117	0.0720	0.4137	2.0275	0.0262
1500	23	mean	356	0.0917	1.9114	2.5777	16.3848	0.1394
		sd	27	0.0261	0.0780	0.2776	1.7744	0.0288
5000	24	mean	370	0.1366	1.9233	2.8124	17.2591	0.1423
		sd	26	0.1607	0.1107	0.5326	2.0969	0.0236
15000	23	mean	365	0.0903	1.8950	2.7660	*18.0724	0.1358
		sd	24	0.0124	0.2331	0.2616	1.2434	0.0359

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Organ Weight (g) Pituitary	Spleen	Thymus	Thyroid	Uterus
0 (Control)	22	mean	358	0.0121	0.6922	0.1853	0.1024	0.6166
		sd	24	0.0035	0.1051	0.0642	0.0212	0.1765
1500	23	mean	356	0.0137	0.6453	0.1803	0.0948	0.6166
		sd	27	0.0039	0.0892	0.0448	0.0192	0.1427
5000	24	mean	370	0.0136	0.7240	0.1876	0.1063	0.5778
		sd	26	0.0045	0.1511	0.0601	0.0164	0.1694
15000	23	mean	365	0.0131	0.6509	0.1651	0.0937	0.5225
		sd	24	0.0028	0.0886	0.0582	0.0120	0.1291

sd=standard deviation
*=significantly different from control group p<0.05

Table B.6.6.1/01-21: relative organ weights for F0 females- selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%) Adrenals	Brain	Kidneys	Liver	Ovaries
0 (Control)	26	mean	349	0.0235	0.5634	0.6977	4.3103	0.0372
		sd	20	0.0039	0.0365	0.0548	0.2864	0.0084
1500	27	mean	352	0.0230	0.5581	0.7233	4.3027	0.0378
		sd	27	0.0045	0.0457	0.0560	0.3435	0.0082
5000	27	mean	364	0.0220	0.5414	0.7062	4.3570	0.0367
		sd	26	0.0032	0.0428	0.0592	0.2810	0.0062
15000	26	mean	362	0.0228	0.5483	**0.7490	***4.6806	0.0340
		sd	28	0.0027	0.0522	0.0521	0.2977	0.0057

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%) Pituitary	Spleen	Thymus	Thyroid	Uterus
0 (Control)	26	mean	349	0.0043	0.1718	0.0465	0.0137	0.2601
		sd	20	0.0010	0.0222	0.0154	0.0023	0.1361
1500	27	mean	352	0.0040	0.1721	0.0508	0.0143	0.2468
		sd	27	0.0011	0.0241	0.0190	0.0036	0.1365
5000	27	mean	364	0.0042	0.1656	0.0500	0.0159	0.2585
		sd	26	0.0009	0.0237	0.0131	0.0026	0.1309
15000	26	mean	362	0.0042	0.1614	0.0436	0.0156	0.2107
		sd	28	0.0008	0.0228	0.0146	0.0021	0.1517

sd=standard deviation
**=significantly different from control group p<0.01
***=significantly different from control group p<0.001

Table B.6.6.1/01-22: relative organ weights for F1 females- selected (group mean values)

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)				
				Adrenals	Brain	Kidneys	Liver	Ovaries
0 (Control)	22	mean	358	0.0253	0.5441	0.7483	4.5970	0.0356
		sd	24	0.0031	0.0283	0.1070	0.4038	0.0071
1500	23	mean	356	0.0258	0.5401	0.7257	4.6047	0.0394
		sd	27	0.0072	0.0396	0.0647	0.2858	0.0081
5000	24	mean	370	0.0372	0.5214	0.7585	4.6543	0.0387
		sd	26	0.0443	0.0416	0.1229	0.3628	0.0078
15000	23	mean	365	0.0249	0.5198	0.7578	**4.9591	0.0371
		sd	24	0.0038	0.0646	0.0517	0.3130	0.0092

Dietary Concentration (ppm)	Number of Animals		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%)				
				Pituitary	Spleen	Thymus	Thyroid	Uterus
0 (Control)	22	mean	358	0.0034	0.1930	0.0518	0.0288	0.1728
		sd	24	0.0010	0.0248	0.0178	0.0064	0.0513
1500	23	mean	356	0.0039	0.1814	0.0506	0.0267	0.1751
		sd	27	0.0012	0.0204	0.0115	0.0051	0.0447
5000	24	mean	370	0.0037	0.1950	0.0509	0.0289	0.1570
		sd	26	0.0012	0.0349	0.0161	0.0055	0.0495
15000	23	mean	365	0.0036	0.1789	0.0454	0.0258	0.1440
		sd	24	0.0007	0.0254	0.0164	0.0039	0.0374

sd=standard deviation
**=significantly different from control group $p<0.01$

Organ weights offspring

There were no toxicologically significant intergroup differences detected for the brain, spleen or thymus for offspring of either sex from either generation. Furthermore, there were no differences in uterus weights for treated females from either generation when compared to controls.

Lower thymus weights were observed for F2 males treated with 15000 ppm when compared to controls ($p<0.05$), however the difference was only seen in the absolute weight and was not reflected in the relative weights. This reduction, in isolation, was considered to be of no toxicological importance according to study author.

Table B.6.6.1/01-23: Organ weights for male offspring- F2 generation (group mean values)

Dietary Concentration (ppm)	Number of Litters		Bodyweight (g) at Terminal Kill	Organ Weight (g) Brain	Spleen	Thymus
0 (Control)	22/21•	mean	52.4	1.3921	0.2459	0.2207
		sd	9.8	0.1306	0.1016	0.0468
1500	23	mean	48.9	1.3054	0.2334	0.2057
		sd	6.7	0.1138	0.0591	0.0427
5000	24/23□	mean	51.4	1.4016	0.2405	0.2136
		sd	7.9	0.1146	0.0860	0.0459
15000	23	mean	46.0	1.3440	0.2049	*0.1766
		sd	6.8	0.1343	0.0568	0.0458

Dietary Concentration (ppm)	Number of Litters		Bodyweight (g) at Terminal Kill	Relative Organ Weight (%) Brain	Spleen	Thymus
0 (Control)	21	mean	52.4	2.7259	0.4647	0.4354
		sd	9.8	0.4870	0.1105	0.0650
1500	23	mean	48.9	2.7130	0.4715	0.4202
		sd	6.7	0.3977	0.0955	0.0660
5000	24/23□	mean	51.4	2.7709	0.4575	0.4158
		sd	7.9	0.4023	0.1189	0.0692
15000	23	mean	46.0	2.9684	0.4399	0.3826
		sd	6.8	0.4321	0.0848	0.0804

sd=standard deviation
*= significantly different from control group $p<0.05$
•=n=21 for terminal bodyweight
□n=23 for spleen weight

Sperm assessments

There were no toxicologically significant effects on the concentration, motility or morphology of samples of sperm from treated F0 and F1 generation males when compared to their controls. Furthermore, no abnormal sperm were detected in the control and treated males from either generation.

There were no toxicologically significant differences in homogenisation resistant spermatid counts for males treated with 15000 ppm from either the F0 or the F1 generation. The number of homogenisation resistant spermatid present in the cauda epididymis for F0 males treated at 15000 ppm were significantly lower than the controls.

Table B.6.6.1/01-24: Sperm assessment and morphology- group mean values (F0)

	Dose (ppm)	**0**		**1500**		**5000**		**15000**	
#Animals (No. of males used for sperm concentration only)		28(24)		27(23)		28(27)		28(22)	
Concentration (M/mL)		1145.8±561.6		1288.5±549.0		1124.5±475.5		1291.4±551.7	
Motile sperm (%)		77±12		81±11		77±11		82±13	
Progressively motile sperm (%)		3±3		4±4		3±3		5±5	
Cauda epididymis	Sperm count (10^6/g	399.9±151.2		-		-		309*±162.6 (23%)	
Testis	Sperm count (10^6/g)	42.1±12.9						41.3±11.2	
Sperm morphology		#	%	#	%	#	%	#	%
	Normal	200	100%	200	100	200	100	200	100
	Decapitate	0	0	0	0	0	0	0	0

	Abnormal	0	0	0	0	0	0	0	0

*Significantly different from the control group p<0.05

Table B.6.6.1/01-25: Sperm assessment and morphology-group mean values (F1)

	Dose (ppm)	**0**		**1500**		**5000**		**15000**	
#Animals (No. of males used for sperm concentration only)		24(23)		24		24		24	
Concentration (M/mL)		873.8±469.6		1008.3±472.7		1229.7±573.2		872.3±598.5	
Motile sperm (%)		59±20		69±14		70±14		59±23	
Progressively motile sperm (%)		2±2		3±2		3±3		2±2	
Cauda epididymis	Sperm count (10^6/g	445.9±213.1		-		-		417.0±190.5 (6%)	
Testis	Sperm count (10^6/g)	39±13.6						40.2±5.4	
Sperm morphology		#	%	#	%	#	%	#	%
	Normal	200	100	200	100	200	100	200	100
	Decapitate	0	0	0	0	0	0	0	0
	Abnormal	0	0	0	0	0	0	0	0

Oocyte assessment

There were no toxicologically significant differences in follicle numbers for F1 females treated with 15000 ppm when compared to controls.

A statistically significant higher number of large follicles (38%) was detected for 15000 ppm females (p<0.01) when compared to control, however, in the absence of any supporting data retrieved from the uterine examinations or any differences in number of offspring produced at this treatment level, this increase was indicative of normal biological variation and of no toxicological importance according to study author.

Table B.6.6.1/01-26: Oocyte assessments-group mean values (F1)

Dietary Concentration (ppm)	Number of Animals Examined		Ovary 1			Ovary 2			Overall		
			Small Follicle	Medium Follicle	Large Follicle	Small Follicle	Medium Follicle	Large Follicle	Small Follicle	Medium Follicle	Large Follicle
0 (Control)	10	mean	4.3	2.5	8.7	4.5	2.4	7.2	4.4	2.4	8.0
		sd	1.9	1.7	3.7	1.9	0.8	1.8	1.6	1.1	2.4
15000	10	mean	6.1	2.9	10.9	5.5	2.8	11.1	5.8	2.9	**11.0
		sd	2.3	1.0	1.8	2.9	1.0	2.8	2.3	0.9	1.5

**=significantly different from control group p<0.01
sd=standard deviation

Histopathology

No treatment-related changes were detected in the F0 generation animals.

In the F1 generation cortical vacuolation of the adrenal glands was observed with a lower incidence and with generally lower grades of severity among males treated with 15000 ppm ($p < 0.05$), 5000 ppm ($p < 0.05$ - 0.01), and 1500 ppm ($p < 0.1$ - 0.05) when compared to controls. The group distribution of incidence and of severity grades may also suggest a consequence of treatment. However, the absence of a dose-related response, may suggest that a higher than normal background incidence of the condition among control male rats may have contributed to the effect on this occasion.

All remaining morphological changes were those commonly observed in laboratory maintained rats of the age and strain employed and, since there were no differences in incidence or severity between control and treatment groups, all were considered to be without toxicological significance.

Table B.6.6.1/01-27: Incidence of adrenal cortical vacuolation in males at terminal kill

	Historical control data	Dietary concentration (ppm)							
		0		1500		5000		15000	
Generation	--	F0	F1	F0	F1	F0	F1	F0	F1
Animals examined	234	28	24	27	24	28	24	28	24
adrenal cortical vacuolation									
Absent	153	20	7	--	14	--	16	16	14
Present	81	8	17	--	10*	--	8**	12	10***
Minimal	57	6	10	--	6	--	6	8	7
Slight	23	2	7	--	4	--	2	4	2
moderate	1	0	0	--	0	--	0	0	1
% present	34.6	29%	71%	--	42%	--	33%	43%	42%

* significantly different from control group $p < 0.1 – p < 0.05$
** significantly different from control group $p < 0.01 – p < 0.05$
*** significantly different from control group $p < 0.05$

CONCLUSION (STUDY AUTHOR)
The oral administration of glyphosate technical to rats by dietary admixture at a maximum dose level of 15000 ppm for two successive generations resulted in possible treatment-related changes at 15000 ppm. The effects however were considered not to represent an adverse health effect, therefore the NOAEL was considered to be 15000 ppm for adult toxicity for both the F0 and F1 generations.

The NOAEL for reproductive and developmental toxicity, for both generations and offspring was considered to be 15000 ppm.

Assessment and conclusion by applicant:
In this study at the highest dose level of 15000 ppm increased organ weights in liver (F0 and F1 females) and kidneys (F0 females) were observed. The study authors stated that there is no toxicological concern regarding the significantly increased liver weights due to the absence of any histopathological changes in the liver. In fact, in the present study no evidence for histopathological examination of the liver was given. At this high dose level a significant decrease in homogenisation-resistant spermatids (HRS, cauda epididymis) was counted in F0 males (control: 400 million/gram; 15000 ppm: 309 million/gram**). Furthermore, in F1 male offspring sexual maturation (preputial separation) was delayed at 15000 ppm without any additional developmental retardation (e.g. body weight). The authors of the study considered this finding in F1 males (45.9 days versus control 43.0 day) to be unrelated to treatment, because no effects on sexual maturation were evident for females and there were no differences in mating performance. Sperm changes and histopathological examinations did not reveal any changes in the testis or epididymes. Although, the later onset of preputial separation in male offspring at 15000 ppm had obviously no impact on reproductive performance in week 29, a treatment-related effect on sexual maturation at a parental-toxic dose cannot be fully excluded. Therefore, the NOAEL for parental, reproductive and offspring toxicity is considered to be 5000 ppm (approx. equivalent to 351 mg/kg bw/day).

Assessment and conclusion by RMS:
In this study, groups of 28 male and 28 female (F0) parents Sprague-Dawley Crl:CD (SD) IGS BR strain rats were fed diet containing 0, 1500, 5000 or 15000 ppm glyphosate technical (F0 generations premating period: 0, 99.4, 292.6, 984.7 mg/kg bw/day for males and 0, 104.4, 322.8 and 1953.3 mg/kg bw/day for females; F1 generation premating period: 0, 116.5, 351 and 1161 mg/kg bw/day for males, and 0, 123.3, 370.8 and 1218.1 mg/kg bw/day for females). After 10 weeks, the animals were mated and allowed to rear the F1 litters to weaning. The regime was repeated with the F1 parents (24 males and 24 females for each group). Pregnant F1 females were allowed to give birth and maintain their offspring on the appropriate diet until Day 21 *post partum*. Effects in adult animals and male offspring were observed at the maximum dose level of 15000 ppm. The NOAELs set in previous evaluation RAR (2015) remains.

Treatment was associated with adverse effects as follows:

- Increased liver weights observed in adult females of both generations at 15000 ppm (F0 females:

absolute weight: ↑13%, relative weight: ↑8%; F1 females: absolute weight: ↑10%, relative weight: ↑8%). RMS considers the magnitude of liver weight changes (increase of 13%) as an adverse effect in the absence of clinical chemistry investigations in the study.

- Increased kidney weights observed in F0 females at 15000 ppm (absolute weight: increased 11%, relative weight: increased 7%)
- Lower number of homogenisation resistant spermatid present in the cauda epididymis observed in F0 generation males at 15000 ppm (309 million/gram compared to 400 million/gram in control).
- Delayed sexual maturation (preputial separation) observed in F1 male offspring at 15000 ppm (Days at completion: 45.9 compared to 43.0 in control). Although, the later onset of preputial separation in male offspring at 15000 ppm had no impact on reproductive performance in week 29, a treatment related effect on sexual maturation at high dose level cannot be excluded.

The NOAEL for parental toxicity was set at 5000 ppm based on increased liver and kidney weights observed in females of both generations at 15000 ppm.

The NOAEL for offspring was set at 5000 ppm based on delayed sexual maturation (preputial separation) observed in F1 male offspring at 15000 ppm.

The NOAEL for reproductive toxicity was set at 5000 ppm based on reduced number of homogenisation resistant spermatid in cauda epididymis observed in F0 generation males at 15000 ppm.

The study is acceptable. The study is performed in accordance with GLP and follows OECD TG 416 (2001).

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- Histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311).

Parameters sensitive to but not diagnostic of EATS:
No parameters missing.

B.6.6.1/04

Data point	CA 5.6.1/004
Report author	[redacted].
Report year	2000
Report title	Glyphosate acid: Multigeneration reproduction toxicity study in rats
Report No.	[redacted]/P/6332
Document No.	Not reported
Guidelines followed in study	OECD 416, Annex V 67/548/EEC, 9.ATP 87/302/EEC OJEC, L133, 47-50 (1988), US-EPA OPPTS 870.3800 (1998)
Deviations from current test guideline	• Anogenital distance not examined as no treatment-related differences in sex ratio and sexual maturation were observed • thyroid weight not recorded • pre-implantation loss was not determined • pup development investigations restricted to body weight, vaginal opening and preputial separation. Comment by RMS: Following deviation was noted in addition: • no individual animal data presented in study report
Previous evaluation	Yes, evaluated and accepted in RAR (2015)
GLP/Officially recognised	Yes, conducted under GLP

testing facilities	
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material: Glyphosate acid, technical
Lot/Batch#: Lot/Batch#: Y04707/082
Purity/Radiochemical purity: Purity: 97.6% (w/w)
Vehicle: Plain diet
Animals: Species: Rat
Strain: Strain: Alpk:APfSD (Wistar-derived)
Age: at least 5 weeks old
Sex: males and females
Weight at dosing: males: approx. 160 g; females: approx. 140 g
Acclimation period: At least 14 days
Diet/Food: CT1 diet (Special Diet Services Ltd., Witham, Essex, UK), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Rats were house in pairs (same sex) in multiple rat racks (with rats of the same group in adjacent cages). During mating animals were house one male: one female. Mated females were housed individually during gestation and lactation and provided with bedding material. After day 29 females separated from their litter were house in pairs until termination. Males were housed up to four per cage after being used for mating.
Environmental conditions:
Temperature: 22 ± 3 °C
Humidity: 30-70%
Air changes: at least 15/hour
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In a two-generation reproduction study groups of 26 male and 26 females (F0 parents) Alpk:APfSD (Wistar-derived) rats were fed diet containing 0, 1000, 3000 or 10000 ppm glyphosate acid. The dose levels were chosen based on results of a previously conducted 2–year dietary toxicity study (reference not stated by study author) conducted in the laboratory in question using the Alpk:APfSD strain of rat.

After 10 weeks of treatment pairing of animals within each dose group was undertaken on a one male: one female basis, to produce the F1 litters. On day 29 *post partum*, groups of twenty-six male and twenty-six female offspring from each dose group of the F0 generation were selected to form the F1 generation. F0 males were terminated after the completion of littering and females were terminated on or soon after day 29 of lactation. Unselected offspring were terminated at day 29 *post partum*. The offspring selected for the F1 generation were dosed for at least 10 weeks and then paired within each dose group to produce the F2 litters. F2 litters were weaned off on day 29 *post partum* and terminated thereafter.

Summary of dose received (mg/kg/day) during pre-mating period-F0 and F1 parents:

F0 parents				
		1000 ppm	3000 ppm	10000 ppm
Males Overall (week 1-10)	mean	99.4	292.6	984.7
Females Overall (week 1-10)	mean	104.4	322.8	1054.3
F1 parents				
		1000 ppm	3000 ppm	10000 ppm
Males overall (week 1-10)	mean	116.5	351.8	1161.0
Females overall (week 1-10)	mean	123.3	370.8	1218.1

Summary of dose received (mg/kg/day) during gestation and lactation:

Gestation				
		1000 ppm	3000 ppm	10000 ppm
F0 parents Litter A Overall (week 1-3)	mean	88.6	271.3	889.0
F1 parents Litter A Overall (week 1-3)	mean	92.1	284.5	932.7
Lactation				
		1000 ppm	3000 ppm	10000 ppm
F0 parents Litter A es overall (week 1-4)	mean	218.6	724.1	2373.0
F1 parents Litter A overall (week 1-4)	mean	237.1	780.6	2476.6

Diet preparation and analyses
For preparation of diet mixtures (60 kg) a known amount of the test substance was mixed with a small amount of basal diet in a mortar using a pestle. Further milled diet was added to give a pre-mix of 1000 g. Each pre-mix was grounded at a constant speed for 15 min with an automatic pestle and mortar. This pre-mix was then added to a larger amount of basal diet and blended for further 6 minutes in a Pharma Matrix Blender Model PMA 150S (TK Fielder). Control diet was treated in the same way but without addition of the test substance. The stability and homogeneity of the test material in diet were determined in the lowest and the highest dose. Dietary admixtures were analysed for achieved concentration at a 2-month interval.

Clinical observations
A check for clinical signs of toxicity, ill-health or behavioural changes was made once daily. A detailed examination of each rat was made at the same time that it was weighed.

Body weight
The initial weights for the F0 adults were recorded immediately prior to feeding the experimental diets for the first time. The initial weights for the F1 adults were recorded at selection on day 29 *post partum*. The bodyweight of each rat was recorded at weekly intervals throughout the pre-mating period. After the pre-mating period the males in each generation were weighed weekly. The female rats were weighed on days 1, 8, 15 and 22 of gestation (the day on which sperm was detected in a vaginal smear was designated day 1 of gestation) and on days 1, 5, 8, 15, 22 and 29 *post partum* (the day of birth was designated day 1 *post partum*). F1 animals were weighed on the day of preputial separation/vaginal opening. All rats were weighed at termination.

Food consumption and compound intake
Food consumption for each cage was recorded throughout the pre-mating period and calculated on a weekly basis. Food utilisation was calculated as the body weight gained by the rats in the cage per 100 g of food eaten. Food consumption was also recorded for females during gestation and lactation and calculated on a weekly basis.

Reproduction parameters
Oestrus cycle
Prior to pairing of females for the F0 and F1 mating phases, a vaginal smear was taken daily for twenty-one days and examined microscopically to determine the stage of oestrous. A vaginal smear was also taken and examined from all F0 and F1 females at termination.

Reproductive performance
The success of mating (production of viable litter) was established. Length of gestation was measured in days from the date of the positive smear to the date of birth. Pre-coital interval was measured as the number of days from the date of pairing to the date of the positive smear.

Litter data
The following litter data were recorded:
The number of offspring born and the number of offspring alive were counted within 24 h after parturition and thereafter on day 5, 8, 15, 22 and 29 post partum. The sex and the litter weight was also recorded at these times. Any clinical findings were recorded. Litters were examined for dead or moribund pups at least once daily.

Physical and sexual development
All selected F1 offspring were observed for sexual development and the body weight for each individual animal at the time of sexual maturation was recorded. During the pre-mating period the rats were examined daily to establish the age on which vaginal opening/preputial separation occurred.

Sacrifice and pathology
All surviving adult females and surviving offspring, except offspring selected to form the F1 generation, were sacrificed on day 29 *post partum*. Males were sacrificed at completion of the littering. All adult animals and offspring, including those dying during the study, were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded. For F0 and F1 females the uterine implantation sites were counted.

The following organs of F0 males and females from each dose group that were sacrificed at the end of the study were weighed at scheduled termination: adrenal gland, brain, left and right epididymides and cauda, kidneys, liver, ovaries, prostrate, pituitary, seminal vesicles (with coagulating gland and fluids), spleen, testes, uterus (with cervix and oviducts).

The following organs from one male and one female offspring from the F1 pairings were weighed: brain, spleen and thymus.

The following tissues were taken from all animals in all groups and preserved in 10% neutral buffered formol saline except testis and epididymis which were preserved in Bouin´s fixative: abnormal tissue, adrenal gland brain*, cervix, left epididymis and cauda, kidney*, liver*, ovary, pituitary, prostate, salivary gland*, seminal vesicle with coagulation gland, spleen*, left testis, uterus with oviducts, vagina (tissue marked with an asterisk were stored).

Beside all pups killed in extremis (age 18-29 days) 3 male and 3 female per F2-litter were given a macroscopic examination at termination on day 29 *post partum*. One of the 3 pups/sex/litter was used for organ weight determination as described above. Following tissues were stored from these pups: brain, spleen, thymus, salivary gland. None of the tissues from the pups were examined histologically.

The reproductive organs from animals suspected of reduced fertility were processed for histopathological examination. Following reproductive organs were examined: adrenal gland, cervix, left epididymis, right ovary, oviducts, pituitary gland, prostate gland, seminal vesicle with coagulation gland, left testis, uterus, vagina.

Semen assessment
At necropsy of adult F0 and F1, sperm were taken from the right distal cauda epididymis. At least 200 individual sperms were evaluated for motility, motility characteristics, and morphology. The abnormal sperm were classified according to Head (detached, double, abnormal shape, abnormal size or abnormal acrosome), Tail (double, severely coiled//kinked or abnormal size) or Multiple (head and tail) abnormalities. In addition, samples of the right testis of the control and high dose animals were homogenised and examined for homogenisation resistant spermatids.

Evaluation of the oocyte number
Primordial and small growing follicles were quantified in the left ovary of all F1 females from the control and high dose groups. Quantification was done using five 5 µm thick sections cut from the central third of each ovary and taken at least 100 µm apart and as evenly spaced as possible.

Statistics
One or a combination of the following statistical methods were applied for the evaluation of the measured parameters: analyses of variance (ANOVA), analyses of covariance, ANOVA followed by analyses of covariance, as well as ANOVA following the double arcsine transformation of Freeman and Tukey (1950), or ANOVA following a square root formation, or Fisher's Exact Test.

All analyses were carried out in SAS (1996). For Fisher's Exact Tests the proportion in each treated group was compared to the control group proportion. Analyses of variance and covariance, with the exception of pup organ weights, allowed for the replicate structure of the study design.

Least-squares means for each group were calculated using the LSMEAN option in SAS PROC MIXED. Unbiased estimates of differences from control were provided by the difference between each treatment group least-squares mean and the control group least-squares mean. Differences from control were tested statistically by comparing each treatment group least-squares mean with the control group least-squares mean using a Student‘s t-test, based on the error mean square in the analysis.

All statistical tests were two sided.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS

The chemical stability of glyphosate acid in the diet at nominal concentrations of 1000 and 10000 ppm was consistent for at least 6 weeks (at room temperature). Homogeneity of the test substance in the dietary mixture was satisfactory, percentage deviations from the overall mean were within 4%. The mean achieved concentrations of glyphosate acid in the preparations were within 9% of the nominal concentrations and the overall mean concentrations were within 3% of the nominal concentrations.

TEST COMPOUND INTAKE

The group mean achieved dosages are summarised in table below.

Table B.6.6.1/02-01: Group mean achieved dose levels F0 and F1 generation

Group	Dietary concentration	Mean achieved dose level			
	(ppm)	(mg/kg bw/day)			
		Males	Females		
			Maturation	Gestation	Lactation
Control	0	0	0	0	0
Low	1000	108.0 [99.4 / F0] [116.5 / F1]	113.9	90.4	227.9
Intermediate	3000	322.2 [292.6 / F0] [351.8 / F1]	346.8	277.9	752.4
High	10000	1072.9 [984.7 / F0] [1161.0 / F1]	1136.2	910.9	2424.8

MORTALITY

There were no test substance related mortalities.

Seven unscheduled deaths occurred during the study. In the F0 generation one control male was killed for human reasons during week 9 because it was found to have a ruptured eyeball. In the low level dose group one female was killed for humane reasons during week 14 having failed to litter on time, dead foetuses were present in the uterus. In the intermediate level dose group one female was killed in week 14 on gestation day 23 due to difficulties with parturition. In the high-level dose group one female with an imperforate vagina and one male having a subcutaneous mass were killed in week 15 and 18, respectively.

In the F1 generation two control animals were killed in extremis. One male due to an accidental injury in week 2 and one female in week 15 due to difficulties with parturition (one dead foetus present in uterus).

CLINICAL OBSERVATIONS

No treatment-related clinical signs of toxicity were noted.

During the pre-mating period, annular constrictions were visible on the tails of the F0 and F1 male and female rats. Almost all males and approximately half of the females, in all groups, were affected. Scaly tail was also observed in some of the animals. These findings were considered incidental to the administration of glyphosate acid in the diet. Other recorded changes in clinical condition were either isolated occurrences or of an incidence comparable with that of the control group.

These signs were considered unrelated to the test substance, since they were either commonly seen in laboratory rats, or caused by physical injury, or occurred in control and treated rats.

BODY WEIGHT
There was no effect of glyphosate acid on body weight adjusted for initial weight for the F0 rats, males and females, during the pre-mating period.

For the F1 males given 10000 ppm, body weight was slightly lower at week 1, in comparison with the control group. Thereafter, body weights adjusted for initial weight remained lower than the controls for the duration of the pre-mating period and were statistically significant different from week 2 through to week 8.

There was no effect of 10000 ppm on the body weight of the Fl females and no effect of 3000 or 1000 ppm on the body weight of the F1 males or the F1 females. There was no effect of glyphosate acid on body weight adjusted for initial weight for either the F0 or F1 rats during gestation or lactation.

Table B.6.6.1/02-02: Body weight during the pre-mating period-F1 generation (Group mean values)

	body weight (g)							
	Control		**Low**		**Mid**		**High**	
F1 generation	**(0 ppm)**		**(1000 ppm)**		**(3000ppm)**		**(10000 ppm)**	
Week	♂ (n=25)	♀ (n=26)	♂ (n=26)	♀ (n=26)	♂ (n=26)	♀ (n=26)	♂ (n=26)	♀ (n=26)
1	80.2	74	81.1	75.2	78.1	74.2	75.3	73.4
2	130.1	115.4	132.3	115.7	128.6	114.7	127.6* (2%)	115.2
3	188.5	152.6	190.7	154.7	186.5	151.2	183.3* (3%)	152.3
4	246.2	178.3	247.6	180.2	242.8	176.5	237.3** (4%)	179.4
5	300.3	201	304.1	202.7	296.5	199.7	289.5** (4%)	202.1
6	345	219.8	347.5	224.1	334.5*	217.2	328.7** (5%)	218.4
7	377.2	231.7	382.4	237.1	369	228.3	360.5** (4%)	234.4
8	403.6	241.9	410.1	245.1	395.3	237.2	387.0* (4%)	245.6
9	425	250.3	433.3	253.6	416.3	245.1	411.8	252.5
10	443.4	259.7	453.1	263.8	435.1	251.7	431.6	258.1
11	461.7	265.7	471.3	271.2	455.5	258.8	449.7	266.9

FOOD CONSUMPTION, FOOD UTILISATION
There was no effect of glyphosate acid on food consumption for the F0 generation as well as all F1 females and F1 males of the low and intermediate level dose group during the pre-mating period.

Only F1 males of the high-level dose group showed significantly lower food consumption throughout the pre-mating period. There was no effect of glyphosate acid on food utilisation for the F0 generation, all F1 females and F1 males of the low and intermediate dose group during the pre-mating period. Food utilisation was slightly higher for F1 males given 10000 ppm glyphosate acid, the difference from control being statistically significant for weeks 5-8 only. There was no effect of glyphosate acid on food consumption for either the F0 or F1 rats during gestation or lactation.

Table B.6.6.1/02-03: Food consumption and food utilisation during the pre-mating period - F1 generation males (group mean values)

	Dietary dose level			
	Control	**Low**	**Mid**	**High**
Parameter	**(0 ppm)**	**(1000 ppm)**	**(3000 ppm)**	**(10000 ppm)**
N	13	13	13	13
Food consumption (g/animal/day)				
Week				
1	19.3	19.7	19.0	18.1*
2	27.2	27.8	27.0	25.9*
3	31.1	31.9	30.9	29.3**

Parameter	Dietary dose level			
	Control	Low	Mid	High
	(0 ppm)	(1000 ppm)	(3000 ppm)	(10000 ppm)
N	13	13	13	13
Food consumption (g/animal/day)				
Week				
4	34.6	35.5	33.9	32.6**
5	35.9	36.4	35.8	33.9*
6	36.5	36.6	34.7	33.2**
7	36.1	36.6	34.6	33.2**
8	35.5	36.1	34.1	33.0**
9	34.8	35.5	33.8	32.3**
10	35.5	35.7	34.1	33.0**
Food utilisation (g growth/100 g food)				
Weeks				
1 – 4	28.44	28.22	27.99	28.11
5 – 8	12.35	12.57	12.31	13.25*
9 -10	7.22	7.61	8.24	8.37
Overall (weeks 1 – 10)	16.78	16.93	16.91	17.38
* significantly different from control group p<0.05 ** significantly different from control group p<0.01				

REPRODUCTIVE PARAMETERS

Oestrus cyclicity/Mating Performance/ Fertility and Gestation

For the F0 females given 10000 ppm the number of cycles was slightly higher and for the F1 females mean cycle length was slightly lower, in comparison with the control group. These differences from control were marginal and inconsistent across the generations and were considered not to be treatment related.

Also, mean cycle length was statistically significant reduced for F0 females given 10000 ppm. Furthermore, mean gestation length was statistically significant reduced for F1 10000 ppm-females (22 days compared to 22.3 days for control).

Table B.6.6.1/02-04: Mating, fertility and gestation parameters (F0)

	ppm		0		1000		3000		10000	
Oestrous cycle – prior to mating (F0)	Regular 4 or 5d cycles		15/26 (58%)		14/26 (54%)		19/26 (73%)		13/26 (50%)	
	Irregular cycle (a)		11/26 (42%)		12/26 (46%)		7/26 (27%)		13/26 (50%)	
	Acyclic (b)		0/26		0/26		0/26		0/26	
	Mean number of cycles		4.73		4.46		4.88		5.38* (14%)	
	Mean cycle length		4.26		4.12		4.05		3.94** (7%)	
Pre-coital interval (F0)	1		7/25 (28%)		7/26 (27%)		7/26 (27%)		11/26 (42%)	
	2-4		17/25 (68%)		19/26 (73%)		18/26 (69%)		14/26 (54%)	
	>4		1/25 (4.0%)		0/26 (0.0%)		1/26 (3.8%)		1/26 (3.8%)	
Mating performance (F0)			M	F	M	F	M	F	M	F
	# Paired		26	26	26	26	26	26	26	26
	# Successful mating		23/26 (88.5%)		22/26 (84.6%)		22/26 (84.6%)		24/26 (92.3%)	
Gestation length and # of pups (F0)	# pregnant			23		22		22		24
	Gestation length (days)	<22		1 (4.3%)		0 (0%)		0 (0%)		0 (0%)
		22		14 (61%)		16 (73%)		18 (82%)		20 (83%)

		>22		8 (35%)		6 (27%)		4 (18%)		4 (17%)
	Mean gestation length (days)			22.3		22.3		22.2		22.2
	Pups born live			277/295 (94.9%)		253/261 (96.5%)		261/264** (99.1%)		291/301 (97.2%)
	Mean # of pups born			12.8		11.9		12		12.5
Post-implantation loss				12/307		18/279		24/288*		10/311
Prop. of litters affected				9/23		12/22		15/22		6/24

(a) at least one cycle of two, three, or six to ten days; (b): at least ten days without oestrus

Table B.6.6.1/02-05: Mating, fertility and gestation parameters (F1)

	ppm		**0**		**1000**		**3000**		**10000**	
	mg/kg bw/day		**0**							
Oestrous cycle – prior to mating (F1)	Regular 4 or 5d cycles		23/26 (88.5%)		25/26 (96%)		23/26 (88.5%)		23/26 (88.5%)	
	Irregular cycle (a)		3/26 (11.5%)		1/26 (4%)		3/26 (11.5%)		3/26 (11.5%)	
	Acyclic (b)		0/26		0/26		0/26		0/26	
	Mean number of cycles		4.35		4.46		4.62		4.50	
	Mean cycle length		4.26		4.12		4.05		3.94** (7%)	
Pre-coital interval (F1)	1		5/26 (19%)		6/25 (24%)		2/24 (8%)		3/26 (11.5%)	
	2-4		18/26 (69%)		19/25 (76%)		21/24 (88%)		19/26 (73%)	
	>4		3/26 (12%)		0/25 (0.0%)		1/24 (4%)		4/26 (15.4%)	
Mating performance (F1)			M	F	M	F	M	F	M	F
	# paired		26	26	26	26	26	26	26	26
	# successful mating		22/26 (84.6%)		23/26 (88.5%)		21/26 (80.8%)		25/26 (96.2%)	
Gestation length and # of pups (F1)	# pregnant			22		23		22		25
	Gestation length (days)	22		15 (68%)		19 (83%)		18 (86%)		25** (100%)
		>22		7 (32%)		4 (17%)		3 (14%)		0** (0%)
	Mean gestation length (days)			22.3		22.2		22.1		22.0**
	Pups born live			226/239 95%		254/263 96.3%		251/254** 98.7%		296/304 96.9%
	Mean # of pups born			10.9		11.4		12.1		12.2
Post-implantation loss				6/245		11/274		6/260		11/315
Prop. of litters affected				5/22		7/23		4/21		6/25

(a) at least one cycle of two, three, or six to ten days; (b): at least ten days without oestrus

LITTER DATA

Size and viability

No overt effects of glyphosate acid on pup survival or on litter size during lactation were detected.

In both generations the incidence of whole litter losses was low and similar across all groups. Glyphosate acid treatment did not affect the percentage of post-implantation loss. The proportion of F1A and F2A pups born live was slightly higher in the glyphosate acid groups than in the control group.

There was no effect of glyphosate acid on litter size at birth or during the time of lactation for either the F1A or F2A pups. The proportion of litters with all pups surviving and the proportion of pups surviving during lactation were also unaffected by the treatment. An increased proportion of litters with all pups surviving noted for the F1A litters in the 10000 ppm group in comparison with the control group were not present for the F2A litters

since the F2A controls showed an improvement over the F1A controls. Sex distribution within the litters was not altered by the administration of glyphosate acid. Individual pup body weights were recorded within 24 hours of births and on days 5, 8, 15, 22 and 29 *post partum*. As pups were not individually identified, the data were recorded by sex and litter.

Table B.6.6.1/02-06: Intergroup comparison of whole litter losses

	ppm	0	1000	3000	10000
F1A litter		2/23 (8.7%)	0/22 (0.0%)	1/22 (4.5%)	1/24 (4.2%)
F2A litter		1/22 (4.5%)	1/23 (4.3%)	2/21 (9.5%)	0/25 (0.0%)

Growth and Development

There was no effect of glyphosate acid on pup weight at birth for the F1A or F2A pups. Thereafter, the adjusted mean body weights of the F1A pups in the 10000 ppm group were lower in comparison with the control group. The differences from control were statistically significant for males from day 8 through to day 29, and for females from day 5 through to day 29 (see table below). A similar effect was neither observed for the F2A pups in the 10000 ppm group or for the F1A pups of the low and intermediate dose level groups.

There was no effect of glyphosate acid on total litter weight of either generation. Also, the day of age when preputial separation or vaginal opening occurred in the F1 parents was unaffected by treatment.

Table B.6.6.1/02-07: mean pup body weights (g)– F1A

ppm	**0**	**1000**	**3000**	**10000**	**0**	**1000**	**3000**	**10000**
Sex	**Males**				**Females**			
Age (d)								
1	5.8 ± 0.6	6.1 ± 0.6	6.0 ± 0.8	6.1 ± 0.6	5.4 ± 0.6	5.8 ± 0.6	5.6 ± 0.6	5.7 ± 0.6
N	23	22	22	24	23	22	22	24
5	8.8 ± 1.6	9.2 ± 1.7	9.0 ± 1.4	8.6 ± 1.2	8.7 ± 1.1	8.9 ± 1.8	8.3 ± 1.4	8.1 ± 1.4
Adj. mean	9.2	9.1	8.9	8.5	9.0	8.5	8.4	8.1** (7%)
N	22	22	21	23	21	21	21	24
8	13.3 ± 1.6	13.5 ± 2.7	13.3 ± 1.9	12.8 ± 1.6	13.0 ± 1.6	13.2 ± 2.4	12.4 ± 2.1	12.2 ± 1.5
Adj. mean	13.8	13.4	13.2	12.6* (9%)	13.3	12.8	12.4	12.1** (9%)
N	21	22	21	23	21	20	21	23
15	26.3 ± 2.6	26.2 ± 5.1	25.9 ± 3.6	24.9 ± 2.8	25.7 ± 2.6	25.8 ± 4.4	24.3 ± 3.6	24.0 ± 2.5
Adj. mean	26.8	26.1	25.8	24.6*(8%)	26.1	25.2	24.5	23.8* (9%)
N	21	22	21	23	21	20	21	23
22	42.5 ± 4.8	42.7 ± 8.1	41.7 ± 6.2	39.7 ± 5.6	41.3 ± 4.8	41.4 ± 7.2	39.1 ± 5.9	38.3 ± 5.3
Adj. mean	43.4	42.4	41.4	39.2* 10%)	41.9	40.3	39.4	37.7* (10%)
N	21	22	21	23	21	20	21	23
29	80.3 ± 8.3	79.9 ± 12.1	79.8 ± 10.5	75.4 ± 8.1	76.1 ± 8.0	75.6 ± 9.9	73.5 ± 8.8	70.8 ± 7.4
Adj. mean	81.7	79.5	79.6	74.6* (9%)	77.1	74.0	74.1	69.9** (9%)
N	21	22	21	23	21	20	21	23

Values expressed as group mean ± SD

* statistically significant difference from control group $p<0.05$

** statistically significant difference from control group $p<0.01$

Table B.6.6.1/02-08: mean pup body weights (g)– F2A

ppm	0	1000	3000	10000	0	1000	3000	10000
Sex	**Males**				**Females**			
Age (d)								
1	6.3 ± 0.5	6.3 ± 0.5	6.3 ± 0.6	6.2 ± 0.4	6.1 ± 0.5	5.9 ± 0.6	5.9 ± 0.6	5.8 ± 0.4
N	22	23	21	25	22	23	19	25
5	9.8 ± 1.1	10.0 ± 1.1	9.4 ± 1.0	9.3 ± 0.9	9.6 ± 1.4	9.6 ± 1.3	9.1 ± 1.1	8.9 ± 0.9
Adj. mean	9.7	9.9	9.3	9.5	9.3	9.6	9.1	9.1
N	21	22	19	25	20	21	19	24
8	14.5 ± 1.9	14.8 ± 1.5	13.9 ± 1.6	14.0 ± 1.5	14.3 ± 2.4	14.2 ± 1.9	13.4 ± 1.9	13.4 ± 1.7
Adj. mean	14.3	14.7	13.8	14.2	13.8	14.2	13.4	13.7
N	21	22	19	25	20	21	19	24
15	27.8 ± 4.0	28.5 ± 3.2	26.5 ± 3.8	27.1 ± 3.3	27.6 ± 4.9	27.5 ± 3.8	25.8 ± 4.4	25.9 ± 3.5
Adj. mean	27.4	28.3	26.4	27.5	26.7	27.5	25.8	26.5
N	21	22	19	25	20	21	19	24
22	45.3 ± 7.2	46.7 ± 5.8	43.1 ± 6.6	44.1 ± 5.8	44.4 ± 7.9	44.7 ± 6.3	41.7 ± 7.2	41.9 ± 6.0
Adj. mean	44.5	46.2	43.1	44.9	42.7	44.8	41.8	42.9
N	21	22	19	25	20	21	19	24
29	84.2 ±11.2	86.8 ± 9.8	80.6 ± 10.1	81.5 ± 8.9	80.1 ± 11.3	80.7 ± 10.1	75.5 ±10.1	75.9 ± 8.8
Adj. mean	83.0	86.0	80.6	82.8	77.7	80.6	75.6	77.4
N	21	22	19	25	20	21	19	24

Values expressed as group mean ± SD

Table B.6.6.1/02-09: Sexual maturation – age group mean as developmental landmark (F1A)

Dose	ppm	0	1000	3000	10000
Vaginal opening	Day of age	35.3 ± 1.3	35.3 ± 1.5	35.2 ± 1.6	35.9 ± 1.6
Balano-preputial separation	Day of age	47.1 ± 2.3	46.6 ± 1.7	47.2 ± 1.6	48.0 ± 2.1

Clinical signs
No clinically observable signs of toxicity were noted for offspring from treated animals.

PATHOLOGY
Necropsy
No macroscopic findings that could be attributed to the treatment with glyphosate acid were observed in any animal of the F0 and F1 generation.

The incidence of unilateral pelvic dilatation was slightly higher (9/69) in F2A females in the 10000 ppm group compared with the other groups. Unilateral pelvic dilatation is a very common spontaneous change in the Alpk:APfSD strain of rat. There was no increase in incidence in the F0 or F1 adults or in the F1A pups and, as an isolated observation, it is considered incidental to treatment with glyphosate acid according to study author.

Organ weights
The treatment of rats with glyphosate acid did not affect the weight of the adrenal glands, brain, right cauda epididymis, epididymides, kidney, liver, ovary, pituitary gland, prostate gland, spleen, seminal vesicles, testes or uterus.

For the F0 males given 10000 ppm glyphosate acid, liver and kidney weights adjusted for body weight were statistically significantly greater than in the control group. Similar changes were not observed in the F1 males given 10000 ppm glyphosate acid. Absolute and relative values were comparable with the control group (see table below). The weight changes seen in the liver and kidney of the F0 males were therefore considered not to be treatment related.

For the F0 males given 3000 or 10000 ppm glyphosate acid, brain weight adjusted for body weight was statistically significantly greater than in the control group. Absolute values were comparable with the control group. Similar changes were not observed in the F1 animals. The weight changes seen in the brain of the F0 males were therefore considered to be incidental to treatment.

Table B.6.6.1/02-10: Liver, kidney and brain weights (relative and absolute) of males (group mean values)

Dietary concentration (ppm)	No. of animals		Organ weight (g)					
			Liver		Kidney		Brain	
			Absolute	Relative	Absolute	Relative	Absolute	Relative
			F0 Generation					
0 (Control)	25	mean	19.3	3.4	3.20	0.57	2.11	0.38
		SD	2.6	0.2	0.38	0.04	0.09	0.03
1000	26	mean	19.1	3.5	3.17	0.58	2.12	0.39
		SD	2.3	0.2	0.36	0.04	0.08	0.03
3000	26	mean	18.7	3.5	3.11	0.58	2.12	0.40
		SD	1.9	0.2	0.27	0.03	0.07	0.03
10000	25	mean	19.7	3.6	3.23	0.59	2.13	0.40
		SD	2.7	0.2	0.38	0.04	0.07	0.05
			F1 Generation					
0 (Control)	25	mean	21.4	3.7	3.42	0.6	2.11	0.37
		SD	2	0.3	0.31	0.05	0.07	0.02
1000	26	mean	21.4	3.7	3.45	0.59	2.12	0.37
		SD	3.3	0.4	0.37	0.04	0.07	0.02
3000	26	mean	20.1	3.6	3.32	0.6	2.1	0.38
		SD	2.6	0.3	0.31	0.04	0.07	0.03
10000	26	mean	19.7*	3.6	3.36	0.62	2.1	0.39
		SD	2.3	0.3	0.28	0.04	0.07	0.03

SD standard deviation
* significantly different from control group $p<0.05$

Table B.6.6.1/02-11: Liver, kidney and brain weights (organ weight adjusted for body weight) of males (group mean values)

Dietary concentration (ppm)	No. of animals		Organ weight adjusted for body weight		
			Liver	Kidney	Brain
			F0 Generation		
0 (Control)	25	mean	18.8	3.13	2.09
1000	26	mean	19.1	3.18	2.12
3000	26	mean	19.0	3.16	2.13* (2%)
10000	25	mean	19.8** (5%)	3.25* (4%)	2.13* (2%)
			F1 Generation		
0 (Control)	25	mean	20.9	3.37	2.11
1000	26	mean	20.5	3.35	2.11
3000	26	mean	20.6	3.38	2.10
10000	26	mean	20.5	3.45	2.11

SD standard deviation
* significantly different from control group $p<0.05$
** significantly different from control group $p<0.01$

For the F1A female pups in the 10000 ppm group absolute thymus weight was statistically significantly lower than in the control group. There was no effect of glyphosate acid on the thymus weight of the F2A pups. The observation in the F1A females is therefore considered incidental to treatment with glyphosate acid.

Table B.6.6.1/02-12: thymus weights (relative and absolute) of females-F1A and F2A litters (group mean values)

Dietary concentration (ppm)	No. of animals		Thymus weight	
			Absolute	Relative
F1A litter				
0 (control)	21	mean	0.276	0.356
1000	19	mean	0.255	0.344
3000	20	mean	0.258	0.357
10000	23	mean	0.240*	0.339
F2A litter				
0	20	mean	0.264	0.331
1000	21	mean	0.258	0.315
3000	19	mean	0.252	0.329
10000	24	mean	0.257	0.344

SD standard deviation
* significantly different from control group $p<0.05$

Sperm assessment
In F0 and F1 males no effect of glyphosate acid on the number of sperm, sperm motility parameters or sperm morphology was observed.

Table B.6.6.1/02-13: Sperm assessment and morphology – group mean values (F0)

	ppm	0	1000	3000	10000
# Animals		25	26	26	25
Average Path Velocity (µm/sec)		98.9 ± 8	97.5 ± 10.2	96.8 ± 10.6	99.5 ± 11.6
Straightness (%)		55.7 ± 5.3	56 ± 7.9	57.5 ± 6.6	55.9 ± 6.5
Motile sperm (%)		85.9 ± 8.8	81.5 ± 11.5	83.5 ± 16.1	85 ± 8.5
Cauda epididymis (Right)	Weight (g)	0.258 ± 0.031	0.238**± 0.025	0.247 ± 0.033	0.256 ± 0.024
	Total (10^6)	131 ± 40	112 ± 51	117 ± 44	138 ± 70
	Sperm count (10^6/g)	513 ± 160	469 ± 213	477 ± 182	550 ± 310
Testis	Total (million) sperm	91 ± 12			90 ±15
	Sperm count (10^6/g)	55 ± 7			53 ± 10
Sperm morphology Head (%)	Detached head	1.72 ± 1.47	1.50 ± 1.74	3.67 ± 7.61	1.58 ± 1.59
	Abnormal shaped head	0.11 ± 0.2	0.09 ± 0.22	0.05 ± 0.15	0.10 ± 0.19
Tail (%)	Coiled/Kinked	0.09 ± 0.18	0.07 ± 0.17	0.23 ± 0.36	0.10 ± 0.25
	Abnormal sized tail	0.22 ± 0.27	0.27 ± 0.34	0.14 ± 0.26	0.37 ± 0.55
Sperm (%)	Normal	97.9 ± 1.7	98.1 ± 1.9	95.9 ± 7.8	97.9 ± 2.1
	Abnormal	2.1 ± 1.7	1.9 ± 1.9	4.1 ± 7.8	2.1 ± 2.1

** significantly different from control group $p<0.01$

Table B.6.6.1/02-14: Sperm assessment and morphology – group mean values (F1)

	ppm	**0**	**1000**	**3000**	**10000**
# Animals		25	26	26	26
Average Path Velocity (µm/sec)		92.1 ± 15.5	91.1 ± 22.0	88.1 ± 16.1	88.1 ± 13.1
Straightness (%)		52.1 ± 8.3	49.6 ± 13.0	51.6 ± 10.0	51.1 ± 8.7
Motile sperm (%)		78.1 ± 15.9	82.7 ± 18.8	79.7 ± 14.2	78.8 ± 11.9

	ppm	0	1000	3000	10000
Cauda epididymis (Right)	Weight (g)	0.255 ± 0.034	0.263 ± 0.037	0.259 ± 0.030	0.255 ± 0.031
	Total (10^6)	112 ± 44	129 ± 72	116 ± 50	103 ± 56
	Sperm count (10^6/g)	444 ± 183	503 ± 308	447 ± 177	419 ± 259
Testis	Total (million) sperm	96 ± 14			92 ±20
	Sperm count (10^6/g)	56 ± 7			55 ± 12
Sperm morphology Head (%)	Detached head	0.65 ± 0.48	0.75 ± 0.93	0.80 ± 0.68	0.59 ± 0.51
	Abnormal shaped head	0.17 ± 0.23	0.13 ± 0.25	0.13 ± 0.22	0.02** ± 0.10
Tail (%)	Coiled/Kinked	0.11 ± 0.20	0.04 ± 0.13	0.10 ± 0.24	0.07 ± 0.22
	Abnormal sized tail	0.11 ± 0.24	0.09 ± 0.23	0.07 ± 0.17	0.09 ± 0.24
Sperm (%)	Normal	99.0 ± 0.8	99.0 ± 1.0	98.9 ± 0.8	99.2 ± 0.6
	Abnormal	1.0 ± 0.8	1.0 ± 1.0	1.1 ± 0.8	0.8 ± 0.6

** significantly different from control group $p<0.01$

Oocyte assessment
There was no effect of 10000 ppm glyphosate acid on the number of primordial and small growing follicles in the left ovary of the F1 parent animals.

Table B.6.6.1/02-15: Intergroup comparison of oocyte quantification- F1 parents

	ppm	0	10000
# Animals		26	26
No. of primordial and small growing follicles (mean values)		51.3 ± 18.5	53.6 ± 15.6

Macroscopic findings
No treatment-related changes were detected in F0 or F1 animals.

Histopathology
No treatment-related changes were detected in the F0 and F1 animals.

CONCLUSION (STUDY AUTHOR)
Continuous feeding of diets containing glyphosate acid at concentrations up to and including 10000 ppm had no effect on the propagation of two generations of the Alpk:ApfSD rat. The fertility and reproductive performance of each generation of parental animals and the clinical condition and survival of their offspring were not adversely affected. The only effect of treatment in the study was a reduction in the bodyweight of the F1A pups in the 10000 ppm group with a subsequent reduction in bodyweight of the selected F1 parent males for the duration of the pre-mating period. The dose level of 3000 ppm glyphosate acid is without any toxicologically significant effect in this study.

Assessment and conclusion by applicant:
This study is considered valid in spite of some deviations from the most recent OECD 416 (2001). The oral administration of glyphosate acid to rats by dietary admixture at a maximum dose level of 10000 ppm for two successive generations of the Alpk:APfSD rat resulted in treatment-related changes at 10000 ppm, where a reduction in the body weight of the F1A pups in the 10000 ppm group with a subsequent reduction in body weight of the selected F1 parent males for the duration of the pre-mating period was observed. Therefore, the NOAEL was considered to be 3000 ppm (equivalent to 322 and 459 mg/kg bw/day for males and females, respectively) for maternal and offspring for both the F0 and F1 generations. The NOAEL for reproduction is considered to be 10000 ppm (equivalent to 1072 / 911 – 2425 mg/kg bw/day).

Assessment and conclusion by RMS:
In this study, dietary administration of glyphosate acid to two successive generations of Alpk:APfSD rats at dose levels of up to 10000 ppm did not affect sexual performance and fertility, and there was no indication of a teratogenic effect of the test substance at any of these concentrations. For the renewal the NOAEL for parental toxicity in study set in previous RAR (2015) is proposed to be changed from 3000 ppm to 10000 ppm.

Treatment was associated with adverse effects as follows:

- Reduced body weight (up to 10%) observed in F1A pups at 10000 ppm

The NOAEL for parental toxicity was set at 1000 ppm (985 mg/kg bw/day, mean daily intake of glyphosate during pre-mating phase in F0 males) (highest dose).

The NOAEL for offspring toxicity was set at 3000 ppm (293 mg/kg bw/day, mean daily intake of glyphosate during pre-mating phase in F0 males) based on reduction in the body weight of the F1A pups in the 10000 ppm group.

The NOAEL for reproductive toxicity was set at 10000 ppm (985 mg/kg bw/d, mean daily intake of glyphosate during pre-mating phase in F0 males) (highest dose level).

The study is acceptable. The study is performed in accordance with GLP and follows OECD TG 416 (2001) except for following deviations, these deviations do not invalidate the study:
(i) no individual animal data presented in study report
(ii) anogenital distance not examined as no treatment-related differences in sex ratio and sexual maturation were observed
(iii) the thyroid was not weighed
(iv) preimplantation loss not determined
(v) pup development investigations restricted to body weight, vaginal opening and preputial separation

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:
- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311).
- Anogenital distance

Sensitive to but not diagnostic of EATS:
- Pre-implantation loss
- Number of implantations corpora lutea

B.6.6.1/05

Data point	CA 5.6.1/005
Report author	[redacted]
Report year	1997
Report title	HR-001: A two-generation reproduction study in rats
Report No.	[redacted] 96-0031
Document No.	Not reported
Guidelines followed in study	OECD 416 (1983), US-EPA FIFRA: 40 CFR 160 (1989), EPA Guidelines Subdivision F, 83-4 (1984), Japan MAFF Guideline 59 NohSan No. 4200 (1985)

Deviations from current test guideline	• Number of animals used are not in line with the recommendation by the guideline • pup development investigations restricted to body weight • developmental landmarks (vaginal opening and preputial separation) not examined • anogenital distance was not determined • thyroid weight and histopathology were not determined • pre- and post-implantation loss were not reported • number of corpora lutea was not given • time to mating was not reported Comments by RMS: Following deviations were noted in addition: • spleen not weighed for parental animals • no organ weighed for pups (the guideline recommends brain, spleen and thymus to be weighed) • testes were not used for enumeration of homogenisation-resistant spermatids but cauda epididymal sperm was enumerated (the guideline recommends both testes and epididymides to be used for enumeration of homogenisation-resistant spermatids and cauda epididymides sperm reserves, respectively)
Previous evaluation	Yes, evaluated and accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material: Glyphosate technical, Code: HR-001
Lot/Batch: T-950308
Purity/Radiochemical purity: Purity: 94.61 % (w/w)
Vehicle: Plain diet
Animals: Species: Rat
Strain: Sprague-Dawley; Crj:CD (SD)
Age: 5 weeks
Sex: males and females
Weight at dosing: Males: 132 - 148 g; females: 112 - 126 g
Acclimation period: 7 days
Diet/Food: Certified pulverised feed (MF Mash, Oriental Yeast Co., Ltd), *ad libitum*
Water: Filtered, sterilised well water, *ad libitum*
Housing: During acclimatisation in groups of five per sex in suspended wire-mesh stainless steel cages). During pre-mating, and mating periods animals were housed in groups of 3/sex/cage. During mating one male and one female were housed in aluminium cages with wire-mesh floors and fronts. Mated females were housed individually during gestation and lactation and provided with bedding material. After day 29 females separated from their litter were house in pairs until termination. Males were housed up to four per cage after being used for mating.
Environmental conditions:
Temperature: 22 ± 2 °C
Humidity: 55 ± 10 %
Air changes: at least 15/hour
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In this two-generation reproduction study groups of 24 Sprague-Dawley rats per sex of the F0 generation received daily dietary doses of 0, 1200, 6000 and 30000 ppm HR-001 in diet. The dose levels were chosen based on results of a preliminary reproductive study in Crj:CD (SD) rats (cited but not submitted). After 10 weeks of treatment pairing of animals within each dose group was undertaken on a one male: one female basis, to produce the F1 litters. The day of proved copulation was designated day 0 of gestation. Copulated females were placed individually into breeding boxes with nestle material. The day of completed parturition was designated day 0 of lactation. On day 4 *post partum*, litter sizes were reduced to a maximum of 8 pups, preferable to 4 males and 4 females, and the remaining pups were culled. Weaning was done on day 21 of lactation and all F0 parental animals were sacrificed. Groups of 24 male and 24 female offspring from each dose group of the F0 generation were selected to form the F1 parents. Unselected offspring were sacrificed and subjected to a gross necropsy.

The offspring selected for the F1 generation were dosed for 10 weeks and then paired within each dose group to produce the F2 litters. F2 litters were weaned on day 21 of lactation and terminated together with F1 parental animals. F1 parental rats which failed to produce F2 offspring (10 males and 10 females with normal external genitalia and oestrus cycle) were mated with untreated rats of the same strain and sacrificed thereafter for fertility assessment (reproductive performance).

Preliminary study (cited but not submitted)
In the preliminary study, Crj: CD (SD) rats, 8/sex/group, were given diets containing glyphosate at dose levels of 0, 1000, 3000, 10000, or 30000 ppm during the 3–week-rearing and subsequent breeding periods. In the 30000 ppm group, treatment related adverse effects on parental animals were evident, and the incidence of loose stool was significantly higher than that in the control group. Moreover, decreases in the body weights and food consumption were observed. The adverse effects on the body weights and food consumption were more prominent in males than in females, and statistically significant differences from controls were detected in the mean body weights of males on weeks 5, 6, and 8 of treatment. Necropsy of parental animals at termination of test substance treatment revealed distention of the cecum in males with a statistically significant increase in the incidence. In the 1000, 3000, and 10000 ppm groups, no treatment-related adverse effects on parental animals were detected.

As for the reproductive performance of parental animals, no treatment-related adverse effects were detected in any treated groups. In offspring in the 30000 ppm group, treatment-related adverse effects were evident. The mean body weights of both sexes on days 14 and 21 of lactation in this dose group were lower than those in the control group, and the differences on day 21 of lactation were statistically significant. Necropsy of pups after weaning revealed distention of the cecum with a significantly increased incidence. In offspring in the 10000 ppm group, the following treatment-related adverse effects were also observed: a decrease in the body weights on day 21 of lactation and a low incidence of distention of the caecum. In the 1000 and 3000 ppm group, no treatment-related abnormalities were detected in any pups. Based on these results, the dose level of 30000 ppm was selected for the high-dose level in the present study and 1200 and 6000 ppm for the low- and middle-dose levels, respectively.

Diet preparation and analyses
Diets were prepared monthly during the pre-mating period, and biweekly during the breeding period. For each dose level a specified amount of the test substance was mixed with a small amount of basal diet in a mortar. This pre-mix was stirred into the remaining part of the diet. The diets were stored at about 4 °C in the dark. Analyses for homogeneity were done for each dose level of the first diet preparation. Analyses for achieved concentration were done for all prepared diets.

Clinical observations
A check for clinical signs of toxicity and mortality was made once daily on all F0 and F1 parental animals. A detailed physical examination was performed on males prior to treatment, and weekly during pre-mating and breeding periods and at necropsy. Females were examined prior to treatment, weekly during pre-mating periods and on gestation days 0, 7, 14 and 20, and on days 0, 7, 14 and 21 of lactation, and at necropsy.

Body weight
Individual body weights F0 and F1 male adults were determined prior to treatment, and weekly during pre-mating and breeding periods and at necropsy. F0 and F1 females were weighed prior to treatment, weekly during

pre-mating periods and on gestation days 0, 7, 14 and 20, and on days 0, 7, 14 and 21 of lactation, and at necropsy.

Food consumption and compound intake
Food consumption for each cage was recorded and daily food consumption was calculated. Determination of food consumption was made on a weekly basis during the pre-mating period for males and females and during the breeding period for males. In addition, for females, total food consumption was determined at the following intervals: day 0-7, 7-14, 14-20 of gestation and of days 0-7, 7-14 and 14-21 of lactation.

Compound intakes in parental animals were calculated during the pre-mating periods for each sex on a weekly basis.

Reproduction parameters
Oestrus cycle
The oestrus cycle was checked daily by microscopically examination of vaginal smears. Examinations were done for each female for one week prior to mating until copulation was confirmed.

Reproductive performance
Mating indices for males and females were calculated separately after copulation was confirmed. In addition, fertility and gestation indices, the length of gestation, as well as the number of implantation sites were determined.

Sperm assessment
An assessment of motility and morphology of epididymal sperm was done at necropsy for 10 males per group, which were selected for the organ weight measurement, as well as for males that failed to impregnate females. Sperm number and motility was analyzed by using a computerized videomicrographic analysis system. Sperm morphology was examined microscopically in stained preparations. Sperm numbers were expressed as the number per cauda epididymis and per gram cauda epididymis. Sperm motility represented percentage of motile sperm % motile). Sperm morphology was expressed as percentage normal of 200 sperm observed per animal.

Litter data
Total number of live and dead pups, and the number of males and females per litter were determined on day 0 of lactation. The sex ratio was calculated for each group. Viability indices were determined for each litter on lactation days 0, 4 and 21. Body weights were determined on lactation days 0, 4, 7, 14 and 21.

A check for clinical signs of toxicity and mortality was made once daily during the lactation period on all F1 and F2 pups. A detailed physical examination was done on lactation days 0, 4, 7, 14 and 21.

Sacrifice and pathology
All surviving parental F0 and F1 males and females were sacrificed on day 21 *post partum* and subjected to a gross pathological examination. Animals of all generations that died, were found dead or were killed moribund during the study period were necropsied as soon as possible. The following organs and tissues were preserved: adrenals, aorta, brain, caecum, colon, duodenum, epididymis, eyes, gross lesions, head (incl. nasal cavity, paranasal sinuses, buccal mucosa and ears), heart, ileum, jejunum, kidneys, larynx, liver, lung, contiguous glands, mammary gland, oesophagus, ovaries, pancreas, pharynx, pituitary, prostate, rectum, seminal vesicles and coagulating glands, spleen, stomach, testes, thymus, thyroid/parathyroid, tongue, trachea, urinary bladder, uterus (cornea and cervix) and vagina.

F1 and F2 pups that were not selected on day 4 of lactation were also killed and necropsied on that day. In addition, F1 weanlings that were not selected for parental animals of the F1 generation and all F2 weanlings were necropsied at 22-26 and 21-26 days of their age, respectively. The same organs, as described above, were preserved from one animal per sex per litter of the F1 and F2 weanlings necropsied.

The following organs weights of 10 F0 and F1 males and females from each dose group that were sacrificed at the end of the study, as well as from pairs of parental animals that failed to mate: adrenal gland, brain, epididymides, kidneys, liver, ovaries, prostate, pituitary, seminal vesicles (with coagulating gland and fluids), testes, uterus.

A histopathological examination was performed on the reproductive organs (ovaries, uterus, vagina, testes, epdidymides, seminal vesicles, coagulating glands, prostate) and pituitary of the control and high dose F0 and F1 parental animals that survived until scheduled termination. A histopathological examination of the reproductive organs and pituitary in the low and mid-dose group was only performed on pairs of animals that had failed to produce offspring. In addition, a histopathological examination was performed on organs with significant weight change, and on all organs with gross pathological changes.

Statistics
One or a combination of the following statistical methods were applied for the evaluation of the measured parameters: Bartlett´s test for equality of variance (p=0.05) followed by parametric analyses of variance in one-way classification (p=0.05) or Dunnett´s t-test or Scheffé´s multiple comparison test (p=0.05, 0.01 or 0.001); or Bartlett´s test followed by Kruskal-Wallis test (P=0.05) and Dunnett-type mean rank test or Scheffé-type mean rank test (p=0.05, 0.01 or 0.001). Fisher´s exact probability test (p=0.05, 0.01 or 0.001) and Mann-Whitney´s U-test (p=0.05 or 0.01) were also used.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS
Chemical analyses in a 4-week dose finding study in mice with HR-001 had proved that the test substance was stable in the basal feed for 5 weeks when they were packed in sealed plastic bags and stored in a dark room at room temperature and continuously stable for at least 2 weeks after being released from the bags and left in an animal room.

Homogeneity of the test substance in the dietary mixtures was satisfactory, percentage deviations from the overall mean were within 4%. The mean achieved concentrations of HR-001 in the diet preparations were in the range of 90 – 105% of the nominal and therefore acceptable.

FOOD CONSUMPTION AND TEST COMPOUND INTAKE
F0 and F1 males
In F0 males, mean food consumption at treatment week 13 in the 1200 ppm group was significantly higher than that in the control group. Since there was no such increase observed in the mid- and high-dose groups throughout the study, this change was not thought to be treatment-related.

In F1 males in the 30000 ppm group, mean food consumption at treatment week 4 was significantly lower than that in the control group, but the values on the other treatment weeks in this dose group were comparable to the controls. In the 1200 and 6000 ppm groups, mean food consumption of F1 males was comparable to the controls throughout the study.

F0 and F1 females
In F0 females, the values on treatment weeks 2-4 in the 30000 ppm group were significantly higher than the controls. Inversely, the value on lactation days 7-14 in this dose group was significantly lower than those in the control group. It was unclear these changes were treatment-related or not. In the 1200 and 6000 ppm groups, mean food consumption of F0 females was comparable to the controls throughout the study.

In F1 females in the 1200 and 6000 groups, mean food consumption on lactation days 14-21 were significantly higher than those in the control group. However, these changes were thought to be incidental because no such increase was observed in the highest dose group. In the 30000 ppm group, mean food consumption of F1 females was comparable to the controls throughout the study.

Table B.6.6.1/03-01: Group mean food consumption (g/rat/day) of F0 and F1 parental female rats

Dietary level (ppm)	No. of animals		Lactation day:		
			0-7	7-14	14-21
			F0 parental females		
0 (Control)	24	mean	42.5	62.9	72.8
		SD	6.1	5.4	6.4
1200	24	mean	40.7	60.3	72.6
		SD	5.6	4.8	6.4

6000	24	mean	42.4	61.6	72.0
		SD	4.9	5.1	4.1
30000	24	mean	41.4	58.0*	69.2
		SD	5.4	5.5	5.7
			F1 parental females		
0 (Control)	23	mean	39.9	59.7	64.3
		SD	5.2	4.8	5.8
1200	23	mean	41.4	58.4	70.2*
		SD	4.5	3.9	5.9
6000	21	mean	40.3	57.4	70.7*
		SD	5.4	5.9	5.8
30000	19	mean	43.4	59.4	65.8
		SD	4.4	5.8	7.7

SD standard deviation
* significantly different from control at $p \leq 0.05$

The group mean achieved dosages are summarised in table below.

Table B.6.6.1/03-02: Group mean achieved dose levels F0 and F1-generation

Group	**Dietary concentration**	**Mean achieved dose level**			
	(ppm)	**(mg/kg bw/day)**			
		Males		Females	
		F0	F1	F0	F1
Control	0	0	0	0	0
Low	1200	83.6	91.7	96.9	104.8
Intermediate	6000	417	458	485	530
High	30000	2150	2411	2532	2760

MORTALITY
F0 and F1 males
There were no treatment related mortalities in F0 or F1 males. During the study period, one F0 male and one Fl male in the control groups and one Fl male in the 6000 ppm group showed malocclusion of the incisors, respiratory wheezing, and red sebum. One of these animals (in the 6000 ppm group) also showed distention of the abdomen. These animals were euthanatised within several days after discovery due to unfavorable prognosis. Necropsy revealed a fracture of the facial bones in all cases, suggesting that the alterations were caused by an accident in a cage.

F0 and F1 females
During the study period, no deaths occurred in any females of the F0 and Fl generations.

CLINICAL OBSERVATIONS
F0 and F1 males
There were no treatment-related clinical signs observed in the 1200 and 6000 ppm groups.

At 30000 ppm F0 and F1 parental males exhibited loose stool with incidents during the pre-mating growth and breeding periods of 3/24 and 2/24 for the F0 generation, and of 13/24 and 0/24 for the F1 generation, respectively, with a significant difference in the value for the pre-mating growth period of the F1 generation. Since this finding was not observed in other groups including control, defecation of loose stool was considered to be treatment.

Statistically significant differences were also observed in the incidence of hair loss during the breeding period for F0 males in all test substance groups. However, the occurrence of this change in the treated groups was rather lower than controls and was considered to be incidental.

F0 and F1 females

There were no treatment-related clinical signs observed in the 1200 and 6000 ppm groups. In F0 and F1 parental females, loose stool was observed at 30000 ppm. The incidences during the pre-mating growth period and the lactation and post-weaning period were 1/24 and 6/24 for the F0 generation, and 4/24 and 2/24 for the F1 generation, respectively, with a significant difference in the value for the lactation and post-weaning period of the F0 generation.

Table B.6.6.1/03-03: Observed clinical signs in male rats (F0 and F1 generation)

Clinical sign	Number of male rats affected in dose group (ppm)							
	Pre-mating growth period				Breeding period			
	0	1200	6000	30000	0	1200	6000	30000
F0								
No. of animals examined	24	24	24	24	23	24	24	24
Swelling of the right auricle	0	0	0	0	0	1	0	1
Red sebum	1	0	0	0	0	0	0	0
Lacrima	0	0	0	1	0	0	0	1
Malocclusion	1	0	0	0	0	0	0	0
Wound: head, neck or back	2	0	0	0	2	0	0	0
Hair loss: head, neck, back, etc.	2	1	1	0	6	1*	0**	0**
Soiled fur perianal region	0	0	0	2	0	0	0	1
Loose stool	0	0	0	3	0	0	0	2
Killed in extremis	1	0	0	0	0	0	0	0
F1								
No. of animals examined	24	24	24	24	23	24	23	24
Soiled fur perinasal region	1	1	1	0	0	1	1	0
Red sebum	1	1	2	0	0	2	1	0
Malocclusion	1	1	1	0	0	1	0	0
Distention of the abdomen	0	0	1	0	0	0	0	0
Hair loss: head, neck, back, etc.	1	1	1	0	1	0	2	0
Soiled fur perianal region	0	0	0	4	0	0	0	1
Erosion in the perianal region	0	0	0	2	0	0	0	0
Loose stool	0	0	0	13***	0	0	0	0
Killed in extremis	1	0	1	0	0	0	0	0

* Significantly different from control at $p<0.05$
** Significantly different from control at $p<0.01$
*** Significantly different from control at $p<0.001$

Table B.6.6.1/03-04: Observed clinical signs in female rats (F0 and F1 generation)

Clinical sign	Number of female rats affected in dose group (ppm)							
	Pre-mating growth period				Mating/gestation and Lactation/post-weaning period			
	0	**1200**	**6000**	**30000**	**0**	**1200**	**6000**	**30000**
F0								
No. of animals examined	24	24	24	24	24	24	24	24
Red sebum	0	0	0	0	0	0	1	0
Hair loss: head, neck, back, etc.	2	0	0	1	2/2	0	0	3/3
Mass on the chest	0	0	0	0	0	0	0	1
Scab on the chest	0	0	0	0	0	0	0	1
Loose stool	0	0	0	1	0	0	0	6*
F1								
No. of animals examined	24	24	24	24	23	23	21	19
Red sebum	0	0	0	1	0	1	0	0
Hair loss: head, neck, back, etc.	1	1	1	0	1/2	1/1	1	0
Mass on the chest	0	0	0	0	0	1/1	0	0
Soiled fur perianal region	0	0	0	1	0	0	0	0
Erosion in the perianal region	0	0	0	0	0	0	0	1

Clinical sign	Number of female rats affected in dose group (ppm)							
	Pre-mating growth period				Mating/gestation and Lactation/post-weaning period			
	0	1200	6000	30000	0	1200	6000	30000
Loose stool	0	0	0	4	0	0	0	2

* Significantly different from control at p<0.05

BODY WEIGHT

F0 and F1 males

Mean body weights of F0 and F1 males in the 30000 ppm group were consistently lower than those in the control group from treatment week 1 to the day of necropsy, and the differences from controls at treatment weeks 1-12 and 14 for the F0 generation, and treatment weeks 1-6 for the F1 generation were statistically significant. In the 1200 and 6000 ppm groups, mean body weights of F0 and F1 parental males were comparable to the controls throughout the study.

Table B.6.6.1/03-05: Selected body weights throughout treatment period – F0 and F1 males (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Week				
			0	5	10	14	18
			F0 Generation				
0 (Control)	24[1]	mean	140	366	454	498	531
		SD	4	34	52	60	64
1200	24	mean	140	373	463	516	549
		SD	4	31	44	53	58
6000	24	mean	140	363	456	501	532
		SD	4	24	37	40	44
30000	24	mean	140	341* (7%)	417* (8%)	457* (8%)	486
		SD	4	25	35	39	44
			F1 Generation				
0 (Control)	24[2]	mean	71	351	484	528	558
		SD	6	29	44	46	51
1200	24	mean	73	355	485	532	567
		SD	7	32	54	70	72
6000	24	mean	71	349	487	540	578
		SD	7	23	43	52	55
30000	24	mean	67** (6%)	326** (7%)	464	511	554
		SD	6	24	39	45	46

1 initial group size, reduced to 23 from week 4 onwards
2 initial groups size, reduced to 23 from week 8 onwards
SD standard deviation
* significantly different from control at p≤0.05
** significantly different from control at p≤0.01

F0 and F1 females

There were no significant differences in mean body weights of F0 females in any treatment group when compared to control. In F1 females in the 30000 ppm group, mean body weight on lactation day 0 was significantly higher than that in the control group. In the 1200 and 6000 ppm groups, mean body weights of F1 parental females were comparable to the controls throughout the study.

Table B.6.6.1/03-06: Selected body weights of F0 and F1 females during pre-pairing (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Week		
			0	5	10
			F0 Generation		
0 (Control)	24	mean	119	225	264
		SD	4	12	16

Dietary concentration (ppm)	No. of animals		Body weight (g) at Week		
			0	5	10
1200	24	mean	119	228	268
		SD	4	15	19
6000	24	mean	119	224	268
		SD	4	13	17
30000	24	mean	119	225	263
		SD	4	14	18
			F1 Generation		
0 (Control)	271	mean	66	218	276
		SD	5	13	20
1200	24	mean	67	226	283
		SD	5	16	22
6000	24	mean	66	218	278
		SD	5	19	25
30000	24	mean	63	213	270
		SD	6	16	22

SD standard deviation

Table B.6.6.1/03-07: Body weights of F0 and F1 females during gestation (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Gestation Day			
			0	7	14	20
			F0 Generation			
0 (Control)	24	mean	271	298	331	404
		SD	16	15	19	24
1200	24	mean	273	300	330	401
		SD	19	20	21	25
6000	24	mean	273	301	332	407
		SD	20	18	19	22
30000	24	mean	271	297	325	397
		SD	18	19	20	28
			F1 Generation			
0 (Control)	23	mean	280	308	339	411
		SD	23	22	22	27
1200	23	mean	286	318	349	425
		SD	21	21	22	27
6000	21	mean	282	314	345	416
		SD	29	25	25	31
30000	19	mean	274	310	343	416
		SD	18	19	19	28

SD standard deviation

Table B.6.6.1/03-08: Body weights of F0 and F1 females during lactation (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Day			
			0	7	14	21
			F0 Generation			
0 (Control)	23	mean	288	333	355	327
		SD	21	21	23	18
1200	24	mean	300	334	351	330
		SD	25	23	17	19
6000	24	mean	299	335	353	328
		SD	22	17	17	20
30000	24	mean	301	328	342	339
		SD	22	17	19	20
			F1 Generation			

Dietary concentration (ppm)	No. of animals		Body weight (g) at Day			
			0	**7**	**14**	**21**
0 (Control)	23	mean	310	344	360	326
		SD	26	24	22	24
1200	23	mean	321	350	361	337
		SD	25	23	22	20
6000	21	mean	324	345	360	342
		SD	25	23	25	21
30000	19	mean	325	348	362	349**
		SD	22	18	20	17

SD standard deviation
** significantly different from control at p≤0.01

REPRODUCTIVE PARAMETERS

F0 males and females

Reproductive performance of F0 parental animals was not adversely affected by test substance treatment, and no significant differences were observed in such parameters as percentage of females having normal oestrous cycle, mating index, fertility index, gestation index, duration of gestation, number of implantation sites, and number, motility and morphology of epididymal sperm between the control group and the treated groups.

F1 males and females

In F1 parental animals, reproductive parameters in the treated groups were also comparable to the controls with the exception of gestation index and number of implantation sites, on which some biases were occasionally observed.

The significant higher number of implantation sites at 1200 ppm when compared to control was considered to be unrelated to treatment, since there was no increase noted at 6000 and 30000 ppm.

The fertility indices in the control, 1200, 6000 and 30000 ppm groups were 95.8 (23/24), 95.8 (23/24), 87.5 (21/24) and 79.2% (19/24), respectively, with lower values (not statistically significant) in the 2 higher dose groups.

Among the total of ten F1 females mated with untreated males, only one female in the 30000 ppm group did not undergo pregnancy. Histopathological of this female showed no abnormalities in the reproductive organs and pituitary. Thus, the cause of infertility of this female was not known. The other nine F1 females were proved to have normal reproductive performance. One F1 male in each of the 1200, 6000 and 30000 ppm groups could not successfully impregnate untreated females mated. These 3 males had histopathological abnormalities in the testes and epididymides, and abnormalities in the sperm parameters, as a cause of infertility. However, the other 7 males were proved to have normal reproductive performance. Thus, the majority of F1 males and females which had failed to produce offspring were proved to have normal reproductive performance (see tables below).

Table B.6.6.1/03-09: Reproductive parameters and litter data (F0)

	ppm	**0**	**1200**	**6000**	**30000**
Oestrous cycle – prior to mating (F0)	Regular 4 or 5d cycles	24/24 (100 %)	23/24 (95.8 %)	24/24 (100 %)	24/24 (100 %)
Mating Index	Males	23/23 (100 %)	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)
	Females	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)
	Fertility Index	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)
	Duration of gestation (days)	22.4	22.3	22.1	22.3
	Number of implantation sites	14.9 ± 1.6	14.9 ± 1.6	15.1 ± 1.1	14.8 ± 2.6
	# of pups delivered (mean ± SD)	13.8 ± 1.6	13.2 ± 1.8	14.1 ± 1.4	13.6 ± 2.6
	Sex ratio	0.459	0.491	0.499	0.446

	ppm	0	1200	6000	30000
Viability Index on lactation day	0	97.5	97.9	95.9	97.4
	4	95.5	99.7	97.8	98.9
	21	100.0	98.4	99.5	99.5

Table B.6.6.1/03-10: Reproductive parameters and litter data (F1)

	ppm	0	1200	6000	30000
Oestrous cycle – prior to mating (F1)	Regular 4 or 5d cycles	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)
Mating Index	Males	23/23 (100 %)	24/24 (100 %)	23/23 (100 %)	24/24 (100 %)
	Females	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)	24/24 (100 %)
Fertility Index		23/24 (95.8 %)	23/24 (95.8 %)	21/24 (87.5 %)	19/24 (79.2 %)
Duration of gestation (days)		22.2	22.4	22.2	22.2
Number of implantation sites		13.9 ± 2.1	15.7 ±1.4*	13.6 ±2.5	14.5 ± 2.1
# of pups delivered (mean ± SD)		12.8 ± 2.1	13.7 ± 2.3	13.0 ± 2.7	13.1 ± 2.7
Sex ratio		0.481	0.514	0.551	0.500
Viability Index on lactation day	0	98.8	98.9	98.9	98.1
	4	99.3	99.7	98.7	99.7
	21	100.0	100.0	100.0	99.3

* Significantly different from control at $p<0.05$

Table B.6.6.1/03-11: Sperm assessment and morphology – group mean values (F0)

F0	ppm	0	1200	6000	30000
# animals		10	10	10	10
Sperm motility (%)		77.9 ± 6.8	77.8 ± 9.5	75.7 ± 5.4	81.9 ± 5.9
Sperm count (10^6)	per cauda epididymis	198 ± 22	193 ± 44	206 ± 41	198 ± 15
	per gram cauda epididym.	660 ± 117	631 ± 122	640 ± 128	665 ± 60
Sperm morphology	Normal	97.7 ± 1.3	98.1 ± 1.6	98.0 ± 1.3	98.2 ± 1.2
	Decapitate	n r.	n r.	n r.	n r.
	Abnormal	n r.	n r.	n r.	0

n.r. Not reported.
Values represent mean ± S.D.

Table B.6.6.1/03-12: Sperm assessment and morphology – group mean values (F1)

F1	ppm	0	1200	6000	30000
# animals		10	10	10	10
Sperm motility (%)		74.5 ± 5.3	73.9 ± 6.4	76.0 ± 4.4	74.8 ± 8.8
Sperm count (10^6)	per cauda epididymis	185 ± 37	199 ± 21	215 ± 45	177 ± 28
	per gram cauda epididymis	618 ± 124	673 ± 92	666 ± 117	547 ± 73
Sperm morphology	Normal	96.1 ± 3.7	95.0 ± 3.9	94.0 ± 3.5	95.1 ± 2.9
	Decapitate	0	0	0	0
	Abnormal	0	0	0	0

n.r. Not reported
Values represent mean ± S.D.

LITTER DATA

Number of pups delivered

Mean number of F1 and F2 pups delivered in the 1200, 6000 and 30000 ppm groups were comparable to the controls.

Sex ratio

Sex ratios of F1 and F2 pups in the 1200, 6000 and 30000 ppm groups were comparable to the controls.

Viability index
The viability indices of F1 and F2 pups in the 1200, 6000 and 30000 ppm groups were comparable to the controls.

Body weights
F1 pups:
There were no effects on mean body weight noted in the low- and mid-dose group when compared to controls. F1 pups of both sexes in the 30000 ppm group, showed significantly higher mean body weights on lactation day 0 than the controls. However, mean body weights on days 14 and 21 were significantly decreased when compared to controls.

F2 pups:
There were no effects on mean body weight noted in the low- and mid-dose group when compared to controls during the lactation period. In F2 pups in the 30000 ppm group, mean body weights of both sexes on day 21 of lactation were significantly lower than those in the control group.

Table B.6.6.1/03-13: Mean pup body weights (F1)

ppm	0	1200	6000	30000	0	1200	6000	30000
Sex	Males				Females			
Age (d)								
0	6.7 ± 0.6	6.8 ± 0.5	6.7 ± 0.4	7.2* ± 0.7	6.3 ± 0.6	6.4 ± 0.5	6.4 ± 0.5	6.8* ± 0.6
N	24	24	23	24	24	24	23	24
4	11.6 ± 1.2	11.6 ± 1.2	11.7 ± 1.0	11.6 ± 1.2	11.1 ± 1.2	11.2 ± 1.1	11.3 ± 0.9	11.3 ± 1.2
N	23	24	24	24	23	24	24	24
7	19.5 ± 1.7	19.1 ± 2.0	19.5 ± 1.6	19.3 ± 1.2	18.6 ± 1.8	18.4 ± 1.9	18.8 ± 1.5	18.3 ± 1.6
N	23	24	24	24	23	24	24	24
14	39.5 ± 3.2	39.4 ± 2.6	39.3 ± 2.6	36.6** ± 2.6 (7%)	38.4 ± 3.6	37.9 ± 2.6	38.2 ± 2.2	35.4** ± 2.6 (8%)
N	23	24	24	24	23	24	24	24
21	63.9 ± 4.4	63.8 ± 4.1	62.4 ± 3.7	55.1 ± 3.5*** (14%)	61.0 ± 4.8	60.6 ± 3.9	59.8 ± 3.1	53.2*** ± 4.0 (13%)
N	23	24	24	24	23	24	24	24

Values expressed in group mean ± SD
* statistically significant difference from control group p <0.05
** statistically significant difference from control group p <0.01
*** statistically significant difference from control group p <0.001

Table B.6.6.1/03-14: Mean pup body weights (F2)

ppm	0		1200	6000	30000	0	1200	6000	30000
Sex		Males				Females			
Age (d)									
0	7.0 ± 0.5		6.9 ± 0.6	7.3 ± 0.7	7.1 ± 0.5	6.6 ± 0.5	6.6 ± 0.7	6.8 ± 0.6	6.8 ± 0.6
N	23		23	21	19	23	23	21	19
4	12.0 ± 1.2		12.1 ± 1.5	12.5 ± 1.5	12.5 ± 1.3	11.6 ± 1.2	11.5 ± 1.6	12.0 ± 1.5	12.1 ± 1.1
N	23		23	21	19	23	23	21	19
7	19.8 ± 1.5		20.0 ± 1.9	20.4 ± 2.2	20.6 ± 1.7	18.9 ± 2.0	19.1 ± 2.1	19.6 ± 2.2	19.9 ± 1.4
N	23		23	21	19	23	23	21	19
14	40.1 ± 3.0		39.0 ± 2.8	38.7 ± 2.9	39.1 ± 2.8	38.7 ± 3.5	38.0 ± 2.2	37.5 ± 2.9	38.1 ± 2.9
N	23		23	21	19	23	23	21	19
21	58.6 ±		59.4 ±	58.3 ±	53.1** ±	56.4 ±	57.1 ±	56.2 ±	51.8* ±

ppm	**0**		**1200**	**6000**	**30000**	**0**	**1200**	**6000**	**30000**
Sex		**Males**				**Females**			
	5.1		4.4	4.3	4.4 (9%)	5.5	4.4	4.5	4.2 (8%)
N	23		23	21	19	23	23	21	19

Values expressed in group mean ± SD
* statistically significant difference from control group p<0.05
** statistically significant difference from control group p<0.01

Clinical signs
There were no treatment-related abnormalities noted in F1 and F2 pups of any dose group.

During the lactation period, deaths and loss due to maternal cannibalism occurred in several pups in all groups including the control. However, the incidences in the treated groups were comparable to the control.

PATHOLOGY
Necropsy
F0 and F1 generation:
Necropsy of parental animals of both sexes noted several findings in all groups including the control group. Among these alterations, the incidences of distension of the caecum in F0 and F1 males and females of the 30000 ppm group were significantly higher than those of the controls, and were considered treatment-related. Statistically significant differences from controls were also found in the incidences of hair loss in F0 males of the 1200, 6000 and 30000 ppm groups. However, the values were rather lower than controls and were considered to be incidental. Other findings were low in their incidences and considered not treatment-related.

Table B.6.6.1/03-15: Incidence of distension of the caecum in F0 and F1 parental male rats

	0, 1200, 6000 ppm			**30000 ppm**		
	Terminal kill	Unscheduled death (control group only investigated)	Total	Terminal kill	Unscheduled death	Total
F0 males						
Large intestine: distension of cecum	0	0 (control)	0	21	-	21***
F1 males						
Large intestine: distension of cecum	0	0 (control)	0	19	-	19***

*** statistically significant difference from control group p≤0.001

Table B.6.6.1/03-16: Incidence of distension of the caecum in F0 and F1 parental female rats

	0, 1200, 6000 ppm			**30000 ppm**		
	Terminal kill	Unscheduled death	Total	Terminal kill	Unscheduled death	Total
F0 females						
Large intestine: distension of cecum	0	-	0	24	-	24***
F1 females						
Large intestine: distension of cecum	0	-	0	17	-	17***

*** statistically significant difference from control group p≤0.001

F1 and F2 pups:
Necropsy of stillbirths found on lactation days 0, pups found dead during lactation days 1-4, and pups killed to reduce the litter size on lactation day 4 demonstrated no treatment-related abnormalities in any of the F1 and F2 pups.

During days 5-21 of lactation, only 2 F1 pups in the 1200 ppm group were found dead. Necropsy of these dead pups were not performed due to advanced autolysis.

Necropsy of F1 and F2 weanlings in the 30000 ppm group noted distension of the caecum, suggesting a treatment-related occurrence. In the 1200 and 6000 ppm groups, no treatment-related abnormalities were observed in any of the F1 and F2 weanlings.

Table B.6.6.1/03-17: Incidence of distension of the caecum in F1 and F2 rat weanlings or pups found dead during lactation days 5-21

	0 ppm	1200 ppm	6000 ppm	30000 ppm
F1 pups				
Large intestine: distension of cecum	0 (0)	0 (0)	0 (0)	89 (0)**
F2 pups				
Large intestine: distension of cecum	0	0 (0)	0 (0)	111 (0)**

** statistically significant difference from control group $p<0.01$
Gross pathological examination of weanlings was performed at 21-26 days of age
Figures in parentheses represent the number of pups found dead during lactation days 5-21 and/or post weaning period

Organ weights
F0 and F1 males:
There were no effects in the absolute and relative organ weights in F0 and F1 males of the low- and mid-dose groups. At 30000 ppm relative weights of the liver and kidneys of F0 and F1 males were significantly higher than the control values. These increases were considered treatment-related. In F1 males in the high-dose group, there was also a significant decrease noted in the absolute and relative weights of the prostate. Besides these changes, the relative brain weight of F0 males in the 30000 ppm group was significantly higher than the control value. However, this finding was considered to be the change associated with the low body weights in this group.

F0 and F1 females:
In F0 females, the absolute and relative weights of all organs were comparable between the control and treated groups. In F1 females in the 30000 ppm group, the absolute and relative weights of the liver and kidneys were significantly higher than the controls, and these increases were considered treatment-related.

Significantly higher-than-control value was also observed in the absolute kidney weight in the 6000 ppm group. However, this increase was not considered treatment-related because statistical significance in the difference between the control and 6000 ppm groups disappeared when all F1 females were subjected to the weighing of the kidneys fixed in 10% neutral buffered formalin. The significant lower relative ovarian weight observed in F1 females in the 1200 ppm group was considered to be an incidental finding because no such decrease was observed in the mid- and high-dose groups.

Table B.6.6.1/03-18: Selected organ weights of males

Dietary concentration (ppm)				Organ weight (absolute weights in mg$)							
		bw		Brain		Kidney		Liver		Prostate	
		(g)		absolute	relative	absolute	relative	absolute	relative	absolute	relative
				F0 Generation							
0	mean	538		2197	0.411	1656	0.308	16900	3.13	642	0.1204
	SD	54		111	0.030	184	0.021	2382	0.20	208	0.0389
1200	mean	533		2154	0.406	1631	0.307	16351	3.08	606	0.1141
	SD	43		68	0.025	115	0.028	1094	0.24	159	0.0287
6000	mean	538		2179	0.409	1690	0.316	16568	3.07	707	0.1332
	SD	55		59	0.040	120	0.033	2335	0.19	126	0.0305
30000	mean	473*		2123	0.451*	1617	0.342*	15617	3.29	569	0.1204
	SD	45		142	0.035	178	0.021 (11%)	2410	0.26	94	0.0184
				F1 Generation							
0	mean	569		2219	0.393	1717	0.303	18163	3.19	662	0.1178
	SD	50		46	0.035	150	0.023	2136	0.18	185	0.0355
1200	mean	532		2170	0.413	1694	0.318	17638	3.28	493	0.0931
	SD	66		81	0.049	288	0.035	3655	0.33	85	0.0158
6000	mean	559		2261	0.406	1796	0.322	17509	3.12	582	0.1039
	SD	47		114	0.031	180	0.019	2380	0.22	196	0.0319
30000	mean	567		2217	0.393	1942	0.344*	20523	3.62**	450*	0.0797*

Dietary concentration (ppm)		bw		Organ weight (absolute weights in mg$)							
				Brain		Kidney		Liver		Prostate	
		(g)		absolute	relative	absolute	relative	absolute	relative	absolute	relative
	SD	50		131	0.029	175	0.038 (14%)	2252	0.23 (13%)	153 (32%)	0.0296 (32%)

$ Relative organ weights to body weights are shown as percent of body weights (considering 10 rats in every group)
SD standard deviation
* significantly different from control group p≤0.05
** significantly different from control group p≤0.01

Table B.6.6.1/03-19: Selected organ weights of females

Dietary concentration (ppm)		bw	Organ weight (absolute weights in mg$)							
			Brain		Kidney		Liver		Ovaries	
		(g)	absolute	relative	absolute	relative	absolute	relative	absolute	relative
			F0 Generation							
0	mean	306	2014	0.658	1147	0.374	13491	4.39	61.1	0.0199
	SD	18	110	0.031	120	0.030	1909	0.41	7.3	0.0021
1200	mean	304	1979	0.652	1143	0.376	13219	4.36	66.6	0.0219
	SD	10	66	0.028	74	0.025	1053	0.36	10.3	0.0030
6000	mean	301	1967	0.655	1110	0.369	13090	4.36	60.0	0.0200
	SD	12	82	0.033	84	0.026	1005	0.29	5.9	0.0022
30000	mean	313	2013	0.646	1202	0.385	14009	4.47	64.6	0.0207
	SD	21	73	0.041	125	0.031	1761	0.38	6.3	0.0014
			F1 Generation							
0	mean	314	2035	0.650	1114	0.355	13436	4.28	68.4	0.0218
	SD	19	112	0.050	125	0.028	1598	0.41	7.0	0.0025
1200	mean	326	2026	0.624	1211	0.372	14611	4.49	61.3	0.0189*
	SD	19	64	0.034	99	0.020	1240	0.32	7.6	0.0027
6000	mean	327	2038	0.628	1242*	0.381	15528	4.73	68.0	0.0209
	SD	28	71	0.056	102 (11%)	0.025	2830	0.53	7.6	0.0023
30000	mean	319	2073	0.652	1304	0.409***	16394**	5.13***	69.5	0.0218
	SD	16	94	0.051	108** (17%)	0.030 (15%)	1835 (22%)	0.44 (20%)	7.6	0.0027

$ Relative organ weights to body weights are shown as percent of body weights (considering 10 rats in every group)
SD standard deviation
* significantly different from control group p≤0.05
** significantly different from control group p≤0.01
*** significantly different from control group p≤0.001

Histopathology
F0 and F1 generations:
In all F0 and F1 males and females in the 30000 ppm group, histopathological examinations of the reproductive organs and pituitaries did not indicate any treatment-related alterations.

No treatment-related histopathological alterations were also evident in the following organs in which significant weight changes were detected: kidneys of F1 females in the 6000 ppm group; kidneys of F0 males and F1 males and females in the 30000 ppm group; and liver of F1 males and females in the 30000 ppm group.

DISCUSSION AND CONCLUSIONS BY STUDY AUTHOR
Oral dietary administration of 0, 1200, 6000 and 30000 ppm glyphosate to Sprague-Dawley rats for two successive generations resulted in treatment-related signs of toxicity in parental rats at 30000 ppm. These consisted of defecation of loose stool in F0 and F1 animals of both sexes and decreases in the body weights in F0 and F1 males. Necropsy revealed distention of the cecum and an increase in the liver and kidney weights in F0 and F1 males and females, and a decrease in the prostate weight in F1 males. Although histopathological alterations were not detected, the weight changes observed in these organs were considered to be treatment-related. In addition, in the 30000 ppm group, significantly higher-than-control value was found in the body weights of F1 females on day 21 of lactation. This change was thought to be associated with a retardation of the pup growth (reduced body weight). In the 1200 and 6000 ppm groups, no treatment-related adverse effects were observed in any parental animals in any generation.

Reproductive performance of parental animals was not affected by test substance treatment in any dose groups. The gestation indices of F1 females in the 6000 and 30000 ppm groups were slightly lower than the control. However, this change was not considered to be treatment-related because the majority of the F1 animals which failed to produce offspring were proved to have normal reproductive performance when they were mated with untreated animals.

In offspring, treatment-related adverse effects were observed at the dose level of 30000 ppm. Although the number of pups delivered, sex ratio, and viability indices in the 30000 ppm group were comparable to the controls, a significant decrease in the body weights and a high incidence of distention of the cecum were observed in pups of both sexes. In the 1200 and 6000 ppm groups, no treatment related alterations were detected in any pups of any generations.

Based on these results, it is concluded for parental animals that dietary concentration of 6000 ppm (417-458 and 485-530 mg/kg bw/day, respectively, for males and females) is the no-observed-effect level, and that 30000 ppm (2150-2411 and 2532-2760 mg/kg bw/day, respectively for males and females) is the overt toxic level. The NOEL for reproductive toxicity is 30000 ppm. For offspring, 6000 and 30000 ppm are the NOEL and toxic level, respectively.

Assessment and conclusion by applicant:

Parental toxicity was observed at highest dose of 30000 ppm (> 2000 mg/kg bw/day) only and consisted of loose stool (F0/F1, m/f), reduced body weight (F0/F1, m) caecum distension (F0/F1, m/f), increased liver and kidney weights (F0/F1, m/f), decreased prostate weight (F1). Histopathological alterations were not detected.

Offspring toxicity was observed at highest dose level only and confined to reduced body weight and caecum distension in both sexes. Based on the results the NOAEL for parental and offspring toxicity was considered to be 6000 ppm (417-458 / 485-530 mg/kg bw/day) and 30000 ppm (2150-2411 / 2532-2760) for reproductive toxicity.

Assessment and conclusion by RMS:

In this study groups of 24 male and 24 females Sprague Dawley Crj:CD (SD) rats were fed diets containing glyphosate technical at concentrations of 0, 1200, 6000 or 30000 ppm for two successive generations. The NOAELs set in previous evaluation RAR (2015) remains.

Treatment was associated with following effects:

- clinical signs (loose stool) observed in parental male and female animals of both generations at 30000 ppm.
- reduced body weights observed in parental male animals of both generations at 30000 ppm (F0 males: 8%; F1 males: 7%).
- increased liver weight observed in parental 30000 ppm-animals (F1 males: abs weight: ↑13%; F1 females: abs weight: ↑ 22%, rel weight: ↑ 20%). The magnitude of 13% was considered adverse in the absence of clinical chemistry investigations in the study.
- increased kidney weight observed in parental animals at 30000 ppm (F0 males: rel weight: ↑11%; F1 males: rel weight: ↑14%; F1 females: abs weight: ↑17%, rel weight: ↑15%).
- decreased prostate weight observed in F1 generation males at 30000 ppm (abs and rel weights: ↓32%)
- Lower fertility index observed at mid and high dose level, however without statistical significance. Indeed, most of the F1 animals were proved to have normal reproductive performance after re-mating with untreated animals, but this is not in accordance with current test guidelines: re-mating should be performed with treated males of the same dose group.
- reduced pup weights observed in F1 and F2 litter animals at 30000 ppm (F1 males:14%, F1 females: 13%; F2 males: 9%, F2 females: 8%)
- Distension of caecum in parental animals (F0, F1, both sexes) and offsprings (F1 and F2 litters) observed at 30000 ppm.

The NOAEL for parental toxicity was set at 6000 ppm (417 mg/kg bw/day) based on clinical signs (loose stool,

both sexes), reduced body weights (males), organ weight changes (increased liver and kidney weights (both sexes), reduced prostate weight) and histopathological changes (distension of caecum) (both sexes) observed in both generations at 30000 ppm.

The NOAEL for offspring toxicity was set at 6000 ppm (417 mg/kg bw/day) based on reduced pup weights and distension of caecum observed in both F1 and F2 litters at 30000 ppm.

The NOAEL for reproductive toxicity was set at 6000 ppm (417 mg/kg bw/day) based on lower fertility indices observed for F1 females at high dose level.

The study is acceptable. The study is performed in accordance with GLP and follows OECD TG 416 (2001) except for following deviations, these deviations do not invalidate the study:

(i) testes were not used for enumeration of homogenisation-resistant spermatids but cauda epididymal sperm was enumerated (the guideline recommends both testes and epididymides to be used for enumeration of homogenisation-resistant spermatids and cauda epididymides sperm reserves, respectively)
(ii) thyroid and spleen not weighed
(iii) vaginal opening and preputial separation not examined
(iv) anogenital distance not determined
(v) no organ weighed for pups (the guideline recommends brain, speen and thymus to be weighed)
(vi) pre-and post-implantation loss not reported
(vii) number of corpora lutea not given.
(viii) time to mating not reported

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance

Sensitive to but not diagnostic of EATS:

- number of corpora lutea
- pre- and post-implantation loss
- time to mating

B.6.6.1/06

Data point	CA 5.6.1/006
Report author	[redacted]
Report year	1993
Report title	Two Generation Reproduction Study in Wistar Rats
Report No.	TOXI 885-RP-G2
Document No.	Not reported
Guidelines followed in study	OECD 416 (1983
Deviations from current test guideline	Missing endpoints: • estrous cycle monitoring • pre-coital interval • sperm analysis • organ weights

	• monitoring of physical and sexual offspring development. Deviations from the current version of OECD 416 (2001) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 416. <u>Comments by RMS:</u> Following deviations were noted in addition: • the highest dose level too low • details of achieved concentrations not presented • parental animals were dosed at least 8 weeks before the mating period (the guideline recommend that dosing shall be continued for at least 10 weeks before the mating period) • a quantitative evaluation of primordial follicles not conducted • data for gestation length not presented • no histopathology (only for organs found abnormal in the macroscopical investigation)
Previous evaluation	Yes, evaluated and accepted in DAR (1998), re-evaluated and considered supplementary in RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Supplementary only (See RMS' assessment below)

MATERIALS AND METHODS

Test material: Glyphosate technical
Lot/Batch: Batch No. 60
Expiry date: July 1992
Stability of test compound: More than two years at ambient temperature
Purity/Radiochemical purity: Purity: 96.8 % (w/w)
Vehicle: Plain diet
Animals: Species: Rat
Strain: Wistar rats (Random bred)
Age: 8 weeks
Sex: males and females
Weight at dosing: Males: 160 - 190 g; females: 141 - 160 g Acclimation period: 7 days
Diet/Food: Standard "Gold Mohur" brand powdered rat feed manufactured by M/s Lipton India Limited, Bangalore, India
Water: Deep borewell water passed through activated charcoal filter and exposed to UV rays (Aquaguard on-line water filter cum-purifier manufactured by M/s Eureka Forbes Limited, Bombay, India) was provided in glass bottles *ad libitum*
Housing: Groups of five/three rats of same sex per cage depending on the size of the animals were accommodated in standard polypropylene rat cages (size: L 430 x W 270x H 150 mm) with stainless steel top grill; bedding material (paddy husk) was changed three times per week.
<u>Environmental conditions:</u>
Temperature: 22 ± 3 °C
Humidity: 40-70 %
Air changes: at least 10-15/hour
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In this two-generation reproduction study groups of 30 Wistar rats per sex of the F0 generation were fed diets containing glyphosate technical at concentrations of 0, 100, 1000 and 10000 ppm. Dietary level would correspond to a mean daily compound intake of 7.7, 77 and 770 mg/kg bw/day. [The mean daily intake was not

reported for all dietary levels, but for the low level of 100 ppm a corresponding average value of 7.7 mg/kg bw/d was given in the original report].

After at least 8 weeks of treatment pairing of animals within each dose group was undertaken on a one male: one female basis, to produce the F1 litters (first parental (P0) animals were treated for 10 weeks before mating). The day of proved copulation (vaginal smear) was designated day 0 of gestation. On day 4 *post partum*, litter sizes were reduced to a maximum of 8 pups, preferable to 4 males and 4 females, and the remaining pups were culled.

Weaning was done on day 21 of lactation and all F0 parental animals were sacrificed. Groups of 30 male and 30 female offspring from each dose group of the F0 generation were selected to form the F1 parents.
The offspring selected for the F1 generation were paired within each dose group to produce the F2 litters. F2 litters were weaned on day 21 of lactation and terminated together with F1 parental animals.

Diet preparation and analyses
The required quantities of test compound were weighed and mixed manually with 1.0 kg of powdered rat feed to prepare the premix. The premixes were added to the bulk of remaining quantities of feed and mixed in ribbon mixer. Prepared feed bulks were sampled at different intervals for assaying test compound concentration in experimental diet.

Clinical observations
All animals were observed daily throughout the study and any visible clinical signs were recorded with details on type, severity, time of onset and duration. Any animal found dead or sacrificed in extremis was necropsied and macroscopically abnormal tissues were retained.

Body weight
Males were weighed weekly until termination. Females were weighed weekly during pre-mating, on gestational days 0, 6, 13, and 20 and on days 1, 4, 7, 14 and 21 of lactation.
Offspring were weighed sex-wise as litters on days 1, 4, 7, 14 and 21 *post partum*.

Food consumption and compound intake
Food consumption for each cage of males was recorded weekly until termination. Food consumption of females was recorded weekly during pre-mating and at the following intervals: days 0-6, 6-13, 13-20 of gestation and days 1-4, 4-7, 7-14 and 14-21 of lactation.

Mating procedure and vaginal smears
After the scheduled period of treatment (minimum of 8 weeks), females were paired on a one-to-one basis with males from the same treatment group. Each morning following pairing, a vaginal smear was prepared from each female and examined for the presence of spermatozoa indicating mating. The day on which evidence of mating was found was designated as day 0 of gestation. Once mating had occurred, males were separated from females and vaginal smearing was discontinued.

Females not mated within seven days of pairing were removed and placed with another male of the same treatment group. This was repeated once again, if needed, thus allowing each female a maximum of 21 days to get mated.

Matings of siblings were avoided by pairing males and females of different litters in each generation. Same mating procedure was adopted in Fl animals after 13 weeks of dietary regime.

Pre-coital interval
The time elapsed between initial pairing and detection of mating was noted.

Duration of gestation
The time elapsed between detection of mating and parturition was recorded.

Reproduction parameters
Reproductive performance:
The following reproductive indices were recorded: Male and female fertility index, fecundity index, mean number of implantations, parturition percentage, percentage mortality of pregnant dams, percentage of live pups

born, in females the pre-coital interval (time elapsed between initial pairing and detection of mating) and duration of gestation.

Litter data
Total number of live and dead pups, viability indices (mean viable litter size on day 0, live birth index), litter weight, individual sex and observations on individual pups (if any) were determined within 24 hours after birth. Survival indices were determined on days 2, 4, 7, 14 and 21 of lactation. Body weights were determined on Lactation days 0, 4, 7, 14 and 21. A check for clinical signs of toxicity and mortality was made once daily during the lactation period on all F1 and F2 pups. On day 4 *post partum*, offspring were culled to reduce litter size to eight (four males and four females, wherever possible).

Assessment of development and reproductive performance of progeny
Following weaning, 30 male and 30 female offspring from the Fl litters were selected to form the second generation (P1) parents. When possible, one male and one female were selected from each litter using random numbers within litters after grossly atypical animals were excluded. When litters were insufficient to provide 30 males and 30 females in each group, additional offspring were randomly selected as above from litters already used within each group. The experimental conditions and serial observations made were same as described for the first generation (P0).

Sacrifice and pathology
All surviving parental F0 and F1 males and females and the non-selected weanlings from F1 and all F2 weanlings were sacrificed and subjected to a gross pathological examination. Tissue collection was done for animals of the parent generations only. Animals of all generations that died, were found dead or were killed moribund during the study period were necropsied as soon as possible.

The following organs and tissues were preserved from all F0 and F1 parents of all groups: Ovaries, uterus, vagina, testes, epididymides, seminal vesicles, prostate, coagulation glands, pituitary, adrenals, liver and kidneys. The organs were examined for gross pathological changes and those found abnormal were examined histopathologically.

Females failing to get mated within 21 days and females failing to produce a viable litter by day 25 *post coitum* were necropsied and any macroscopically abnormal tissue was retained for histopathological examination. The presence of corpora lutea, implantations and resorptions was examined in females which had failed to produce a viable litter.

On day 4 *post partum*, offspring were culled to reduce litter size to eight, where possible; culled offspring or found dead were necropsied. All F2 pups were sacrificed at weaning.

Statistics
One or a combination of the following statistical methods were applied for the evaluation of the measured parameters: Dunnett´s t-test (for body weight, food consumption, litter number, litter weight, gestation and lactation period), Z Test (for mating performance, fertility index, gestation index, live birth index, viability index, lactation index, pups survival data, number of dead pups at birth, survival indices, number littered) and t/r test (for dose-response relationship).

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS
In-house stability study for glyphosate technical was carried out at 0, 2000 and 20000 ppm. Chemical stability was given for 30 days at room temperature with a loss of less than 7% at 0, 2000 and 20000 ppm levels in experimental diet when stored in polyethylene lined stainless steel drums.

The mean achieved concentrations of glyphosate in the diet preparations were analysed; the achieved concentrations were in the range of 96-100 % of the nominal and therefore acceptable.

MORTALITY
F0 and F1 males:
There were no mortalities in male animals.

F0 females:
In the females, there were three mortalities, two in the low dose group, (one dystokia and one suppurative pneumonia) and one in the high dose group (cause of death not ascertained).

F1 females:
One dam in low dose group died of dystokia.

CLINICAL OBSERVATIONS
F0 generation:
Nasal discharge and snuffling and cannibalism were seen in all groups. No other treatment related changes in clinical signs were observed.

F1 generation:
The incidence of clinical signs was low and not treatment or dose related. There was no incidence of total cannibalism and the incidence of partial cannibalism was similar in all study groups.

Table B.6.6.1/04-01: Observed clinical signs and mortalities in F0 generation

Clinical sign	Number of rats affected in dose group*							
	Control (0 ppm)		Low (100 ppm)		Mid (1000ppm)		High (10000 ppm)	
	♂	♀	♂	♀	♂	♀	♂	♀
General affections Weakness/loss of body weight	1/30	0/30	3/30	4/30	0/30	0/30	0/30	0/30
Respiratory affections Nasal discharge Snuffing	12/30 7/30	9/30 5/30	22/30 6/30	14/30 5/30	10/30 5/30	12/30 8/30	20/30 8/30	8/30 6/30
GIT affection Soft stool	0/30	0/30	1/30	0/30	0/30	0/30	0/30	0/30
Skin affections Biting injury	0/30	0/30	0/30	0/30	1/30	0/30	0/30	0/30
Urogenital affections Urine incontinency	0/30	0/30	1/30	1/30	0/30	0/30	0/30	0/30
Parturition performance								
Prolonged gestation	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Prolonged parturition	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Mating not confirmed	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Not littered	NA	0/30	NA	4/30	NA	2/30	NA	0/30
Total cannibalism of pups	NA	1/30	NA	0/30	NA	0/30	NA	0/30
Partial cannibalism of pups	NA	3/30	NA	4/30	NA	5/30	NA	4/30
Dystokia death	NA	0/30	NA	1/30	NA	0/30	NA	0/30
Subsequent total cannibalism	NA	1/30	NA	4/30	NA	1/30	NA	1/30
Death during treatment	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Death during gestation	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Death during lactation	NA	0/30	NA	1/30	NA	0/30	NA	1/30
Mortality	0/30	0/30	0/30	2/30	0/30	0/30	0/30	1/30

* x/y number affected / total number of animals in group
NA not applicable for male animals

Table B.6.6.1/04-02: Observed clinical signs and mortalities in F1 generation

Clinical sign	Number of rats affected in dose group*							
	Control (0 ppm)		Low (100 ppm)		Mid (1000ppm)		High (10000 ppm)	
	♂	♀	♂	♀	♂	♀	♂	♀
General affections Weakness/loss of body weight	3/30	1/30	0/30	1/30	0/30	5/30	1/30	3/30

Clinical sign	Number of rats affected in dose group*							
	Control (0 ppm)		Low (100 ppm)		Mid (1000ppm)		High (10000 ppm)	
	♂	♀	♂	♀	♂	♀	♂	♀
Neurological affections								
Circling disease	0/3	0/3	0/3	0/3	0/3	0/3	0/3	2/3
Respiratory affections								
Nasal discharge	0/30	2/30	3/30	4/30	0/30	2/30	2/30	0/30
Snuffing	3/30	4/30	6/30	3/30	2/30	2/30	5/30	4/30
GIT affection								
Soft stool	0/30	0/30	9/30	0/30	10/30	0/30	6/30	0/30
Skin affections								
Alopecia	0/30	0/30	0/30	0/30	0/30	2/30	0/30	1/30
Urogenital affections								
Urine incontinency	0/30	0/30	1/30	0/30	1/30	0/30	5/30	1/30
Parturition performance								
Prolonged gestation	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Prolonged parturition	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Mating not confirmed	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Not littered	NA	4/30	NA	2/30	NA	8/30	NA	2/30
Partial cannibalsm of pups	NA	12/30	NA	7/30	NA	3/30	NA	12/30
Total cannibalism of pups	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Dystokia death	NA	0/30	NA	1/30	NA	0/30	NA	0/30
Subsequent total cannibalism	NA	0/30	NA	0/30	NA	0/30	NA	0/30
Mortality	0/30	0/30	0/30	1/30	0/30	0/30	0/30	0/30

*x/y number affected / total number of animals in group
NA not applicable for male animals

BODY WEIGHT

F0 males:

Initial body weight of treatment groups was higher compared to the control group and this trend continued during the entire treatment period. The absolute weight gain (difference between initial and terminal) during entire treatment period was similar to control group in low and high dose while in mid dose it was slightly higher.

F0 females:

No significant treatment related differences were noted between treated and control groups.

F1 males:

Mid dose group body weight (both initial and in subsequent weeks) was more than control. In high dose group initial body weight (Week 0) was higher than control but at Week 2 and 3 it was less. However, in this group the body weight tended to be higher (not significant) during last seven weeks.

F1 females:

The body weight of all treatment groups at selection (Week 0) was higher than in the control group and continued to be significantly higher than in the control group for up to Week 10 in mid and high dose groups. Body weights of the high dose group dams on Days 0, 6 and 13 of gestation period were significantly higher compared to controls but the body weight gain was statistically not significantly different. Another incidental significant finding was higher body weight (Gestational Day 0-20) of mid dose group dams compared to controls. Absolute body weight of mid dose group on Lactation Days 1 and 4 and that of high dose group during all periods of lactation was significantly higher than in control group. The mid dose group had lost body weight during Days 7-14, 14-21 and 1-21 of lactation period as compared to control.

Table B.6.6.1/04-03: Selected body weights throughout treatment period – F0 and F1 males (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Week			
			0	5	10	16/18*
			F0 Generation			
0 (Control)	30	mean	160	244	285	290
		SD	9.7	18.1	19.0	14.3
100	30	mean	182+	271+	311+	317+
		SD	19.6	19.3	23.8	32.2
1000	30	mean	175+	262+	307+	328+
		SD	19.8	20.3	20.9	22.9
10000	30	mean	190+	282+	312+	321+
		SD	24.3	32.4	36.7	35.6
			F1 Generation			
0 (Control)	30	mean	70	183	279	338
		SD	15.9	25.0	24.2	31.5
100	30	mean	69	206+	293	339
		SD	13.6	22.4	29.9	37.4
1000	30	mean	84+	213+	305+	364+
		SD	13.8	24.5	29.8	29.6
10000	30	mean	72	186	282	353
		SD	10.3	21.9	28.2	32.6

SD - standard deviation
\+ - significantly higher than control
* - values for F0 generation are from week 16, values for F1 generation are from week 18

Table B.6.6.1/04-04: Selected body weights of F0 and F1 females during pre-pairing (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Week			
			0	5	9	12
			F0 Generation			
0 (Control)	30	mean	142	175	199	Not reported
		SD	10.2	9.6	11.9	
100	30	mean	160+	182+	199	Not reported
		SD	12.7	14.5	12.8	
1000	30	mean	142	178	193	Not reported
		SD	11.9	14.6b	19.6	
10000	30	mean	141	186+	200	Not reported
		SD	11.0	17.2	15.9	
			F1 Generation			
0 (Control)	30	mean	66	143	182	197
		SD	15.9	13.4	13.0	15.2
100	30	mean	67	153+	186	195
		SD	13.3	16.2	14.9	15.7
1000	30	mean	69	159+	197+	202
		SD	12.8	12.8	15.4	17.3
10000	30	mean	78+	158+	194+	207
		SD	8.8	15.1	16.6	19.4

SD standard deviation
\+ significantly higher than control

Table B.6.6.1/04-05: Body weights of F0 and F1 females during gestation (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight (g) at Gestation Day			
			0	6	13	20
			F0 Generation			
0 (Control)	30	mean	205	224	244	289
		SD	16.9	15.4	14.2	22.7
100	27	mean	203	218	237	278
		SD	14.0	15.1	17.0	22.1
1000	28	mean	199	216	237	284
		SD	16.6	18.8	23.5	28.9
10000	30	mean	200	218	239	288
		SD	13.3	15.5	19.6	26.5
			F1 Generation			
0 (Control)	26	mean	205	220	242	299
		SD	16.6	17.4	22.0	25.8
100	28	mean	203	219	242	295
		SD	17.8	18.1	20.0	26.7
1000	22	mean	208	226	250	316
		SD	19.0	22.3	20.9	26.9
10000	28	mean	219+	236+	258+	316
		SD	19.5	19.9	21.2	29.3

SD standard deviation
Not reported not applicable
+ significantly higher than control

Table B.6.6.1/04-06: Body weights of F0 and F1 females during lactation (group mean values)

Dietary concentration (ppm)	No. of animals		Body weight Change (g) at Day			
			1	4	14	21
			F0 Generation			
0 (Control)	30	mean	214	229	243	233
		SD	18.0	13.9	19.6	18.5
100	30	mean	214	223	226-	226
		SD	17.2	15.2	25.4	19.9
1000	30	mean	213	227	238	233
		SD	20.4	24.2	25.3	19.0
10000	30	mean	223	230	248	238
		SD	21.2	22.6	19.7	22.9
			F1 Generation			
0 (Control)	26	mean	221	230	249	248
		SD	25.1	20.7	17.2	16.8
100	27	mean	222	233	248	239
		SD	25.9	22.7	19.1	16.2
1000	22	mean	244+	249+	259	244
		SD	21.3	22.0	23.0	27.9
10000	28	mean	244+	251+	268+	269+
		SD	21.4	20.3	24.0	22.0

SD standard deviation
+/- significantly higher/ lower than control

FOOD CONSUMPTION

F0 parents:

Mean food consumption of males was similar to the controls throughout the study. High dose female animals tended to consume significantly more food than controls during gestation. During lactation low and mid-dose females consumed significantly less than controls, especially for the Periods 7-14 and 14-21. High dose females consumed significantly more food for Lactation Days 4-7 as compared to controls.

F1 males:
Treatment groups did not show consistent and dose related changes as compared to control group. However initially (Weeks 0-2) mid and high dose groups consumed significantly less feed and later on a few occasions mid dose group showed increased consumption.

F1 females:
Treatment group dams did not show treatment and dose related consistent difference from control group; on a few occasions the treatment groups showed both increased/decreased food consumption over control. During gestation there was no statistically significant inter group difference in feed consumption between control and treatments during gestation period. Low dose dams consumed significantly less food than controls during different lactation periods (except for Day 7 and Period 7-14). Mid and high dose group dams did not show any treatment and dose related changes over control except for an incidental finding of increased and decreased feed consumption on Day 7, 14 and Period 7-14 and 14-21 respectively in mid dose group.

Table B.6.6.1/04-07: Food consumption throughout treatment period – F0 and F1 males (selected group mean values)

Dietary concentration (ppm)	**No. of animals (No. of cages)**		**Food consumption (g) in Week**			
			0	**5**	**9**	**16/18***
			F0 Generation			
0 (Control)	30 (6)	mean	21.2	18.3	19.0	20.7
		SD	1.47	0.82	1.41	4.27
100	30 (6)	mean	19.2	18.8	18.8	19.7
		SD	2.14	0.75	2.71	2.23
1000	30 (6)	mean	17.2	19.7	21.3	18.2
		SD	0.41	0.82	1.21	1.17
10000	30 (6)	mean	18.8	19.2	19.2	19.0
		SD	0.75	1.33	2.56	1.79
			F1 Generation			
0 (Control)	30 (6)	mean	15.7	17.5	19.0	24.3
		SD	0.88	0.77	0.89	2.01
100	30 (6)	mean	15.9	17.5	22.1	22.8
		SD	0.80	0.63	5.17	1.00
1000	30 (6)	mean	14.3-	18.9	22.5	22.9
		SD	0.52	1.46	2.43	1.14
10000	30 (6)	mean	13.2-	17.8	19.3	23.3
		SD	0.93	1.13	0.88	0.86

SD standard deviation
- significantly lower than control
* values for F0 generation are from week 16, values for F1 generation are from week 18

Table B.6.6.1/04-08: Food consumption of F0 and F1 females during pre-pairing (selected group mean values)

Dietary concentration (ppm)	**No. of animals (No. of cages)**		**Food consumption (g) in Week**			
			0	**5**	**9**	**12**
			F0 Generation			
0 (Control)	30 (6)	mean	21.3	18.3	18.5	Not reported
		SD	1.51	0.82	2.07	
100	30 (6)	mean	19.0	18.0	18.0	Not reported
		SD	5.22	1.67	2.37	
1000	30 (6)	mean	14.0	17.0	17.7	Not reported

Dietary concentration (ppm)	No. of animals (No. of cages)		Food consumption (g) in Week			
			0	5	9	12
		SD	1.41	0.89	2.73	
10000	30 (6)	mean	17.8	18.8	18.8	Not reported
		SD	0.75	1.94	3.25	
			F1 Generation			
0 (Control)	30	mean	15.8	16.9	16.0	22.5
		SD	0.94	1.50	2.10	6.42
100	30	mean	14.0	17.4	26.2+	23.1
		SD	1.30	0.58	8.65	5.01
1000	30	mean	13.8	18.3	22.4+	21.0
		SD	1.03	1.54	5.29	1.35
10000	30	mean	13.5	18.2	19.5	20.7
		SD	1.10	0.68	0.71	0.59

SD standard deviation
+ significantly higher than control

Table B.6.6.1/04-09: Food consumption of F0 and F1 females during gestation (group mean values)

Dietary concentration (ppm)	No. of animals		Food Consumption per Day (g) during Period			
			0-6	6-13	13-20	0-20
			F0 Generation			
0 (Control)	30	mean	17	18	21	19
		SD	4.0	4.0	4.7	3.5
100	27	mean	14-	17	19	17
		SD	3.3	3.2	4.7	2.9
1000	28	mean	18	19	23	20
		SD	4.7	5.1	6.1	4.0
10000	30	mean	22+	23+	22	23+
		SD	9.1	6.1	6.9	5.5
			F1 Generation			
0 (Control)	26	mean	21	20	24	22
		SD	3.5	2.7	3.1	2.6
100	28	mean	21	21	23	22
		SD	4.0	3.5	4.3	3.1
1000	22	mean	22	22	23	25
		SD	4.6	3.6	2.4	2.8
10000	28	mean	21	22	25	23
		SD	5.3	3.8	4.3	3.5

SD standard deviation
+/- significantly higher/ lower than control

Table B.6.6.1/04-10: Food consumption of F0 and F1 females during lactation (group mean values)

Dietary concentration (ppm)	No. of animals		Food Consumption per Day (g) during Period			
			1-4	4-7	14-21	1-21
			F0 Generation			
0 (Control)	28	mean	35	36	55	42
		SD	11.7	9.5	12.3	5.4
100	22	mean	33	37	44-	36-
		SD	13.4	17.5	13.7	7.5
1000	27	mean	30	41	48	37-
		SD	13.8	16.2	9.6	7.2
10000	28	mean	36	49+	59	45

Dietary concentration (ppm)	No. of animals		Food Consumption per Day (g) during Period			
			1-4	4-7	14-21	1-21
		SD	14.9	16.4	13.6	9.8
			F1 Generation			
0 (Control)	30	mean	38	46	60	42
		SD	15.5	15.2	7.3	6.7
100	30	mean	28-	36-	53-	37-
		SD	13.7	8.1	9.4	6.7
1000	30	mean	34	40	50-	39
		SD	11.7	10.7	7.4	4.8
10000	30	mean	30	40	59	41
		SD	6.7	11.2	6.5	3.8

SD standard deviation
+/- significantly higher/ lower than control

REPRODUCTIVE PARAMETERS

Reproductive performance parameters of F0 parental animals such as female fertility index, number of implantations, gestation index, duration of gestation and live birth index were not significantly different between treated and control groups. Male fertility index was significantly higher in low and high dose groups over control.

F0 generation:

On Day 1 of lactation, mean litter size was significantly less than control in low and mid dose groups and the mean viable litter size at birth was significantly less in low dose group; the number of live pups on Day 1 was significantly lower in the mid-dose group.

F1 generation:

Reproductive performance parameters of F1 parental animals such as male and female fertility index, fecundity index, parturition percentage and mortality of pregnant dams was not different between treatment and control groups. The incidence of dams not littered tended to be higher in the mid-dose group compared to controls. A significantly decreased number of implantations was observed in low and mid dose groups; the percentage of live pups born was significantly reduced in the mid dose group and significantly increased in the high dose group.

Table B.6.6.1/04-11: Reproduction data F0 and F1 animals

	Group 1 - control		Group 2		Group 3		Group 4	
	0 ppm		100 ppm		1000 ppm		10000 ppm	
	F0	F1	F0	F1	F0	F1	F0	F1
Number of dams in group	30	30	30	30	30	30	30	30
Number of dams mated	30	30	30	30	30	30	30	30
Number of dams pregnant	30	26	27	27+1*	29	25	30	28
Number of dams littered	29	26	26	27	28	22	30	28
Female fertility index (%)	100.0	86.7	90.0	93.3	96.7	83.3	100.0	93.3
Male fertility index (%)	86.7	86.7	100.0+	86.7	96.7	96.7	100.0+	83.3
Mean number of implantations	12.1	13.4	11.2	11.6-	11.0	12.0-	12.3	12.9
Mean litter size	11.3	11.7	9.8-	10.4	9.9-	10.9	10.4	11.9
Mean viable litter size at birth	11.0	11.7	9.7-	10.4	9.9	10.9	9.9	11.9
Number of pups	320	305	253	281	276-	239	296	334

	Group 1 - control		Group 2		Group 3		Group 4	
	0 ppm		100 ppm		1000 ppm		10000 ppm	
	F0	F1	F0	F1	F0	F1	F0	F1
alive on day 1								
Number of dead pups on day 1	9	0	2	0	0	0	15	0
Percentage of live pups born [%]	87.9	87.6	83.5	86.5	86.5	79.7-	80.0-	92.8+

- significantly decreased; + significantly increased
* one dam died due to dystocia

LITTER DATA

Number of pups delivered

Mean number of F1 and F2 pups delivered and mean litter sizes in the 100, 1000 and 10000 ppm groups were comparable to the controls. For F1 pups the number of live pups on day one was significantly less in mid dose group only. The dose response relationship was not evident.

Sex ratio

Sex ratios of F1 and F2 pups in the 100, 1000 and 10000 ppm groups were comparable to the controls.

Viability index

F1 pups:

In the low dose group the pup survival index for Days 4, 14 and 21 was significantly lower than in controls. In the mid dose group the live birth index and Day 14 survival index were higher and Day 4 survival index was lower compared to controls. In the high dose group on Day 14 and 21 survival index was higher than in controls. Dose response relationship was not seen in these parameters.

F2 pups:

There were no statistically significant inter group differences between control and treatment groups in parameters of F2 litters at first observation including incidence of external abnormalities in pups. The mean number of pups (combined and individual sex) during different periods of lactation did not show statistically significant differences compared to control group.

The group mean values of pup survival data parameters like: live birth index, 24 hours survival index and survival index for Days 4, 7, 14 and 21 did not show any significant inter group difference between control and treatment groups.

Body weights

F1 pups:

Mean litter weight of combined sex and female pups in treatment groups were significantly more than control group on Day 1 and 4, respectively. On Day 7 combined sex litter weight and male pup weight was significantly less than control in low dose group while in high dose group it was more than control group. On Day 21 the mean body weight of complete litter and individual sex pups of mid dose group were more than control group. None of these showed any apparent dose response relationship.

F2 pups:

Combined sex litter weight on day one and that of female pups of all treatment groups was higher than in controls; in addition combined sex litter weight in low and mid dose groups and that of male and female in mid dose group was higher than control on Day 4. In high dose group the male pup body weight on Day 14 and 21 was lower than control. None of these parameters showed any dose response relations.

Clinical signs

There were no treatment-related abnormalities noted in F1 and F2 pups of any dose group.

During the lactation period, deaths and loss due to maternal cannibalism occurred in several pups in all groups including the control. However, the incidences in the treated groups were comparable to the control.

Table B.6.6.1/04-12: Litter data F1 and F2 litters

	Group 1- control 0 ppm		Group 2 100 ppm		Group 3 1000 ppm		Group 4 10000 ppm	
	F1	F2	F1	F2	F1	F2	F1	F2
Number of litters examined	29	26	26	27	28	22	30	28
Mean litter size at birth	11.3	11.7	9.8-	10.4	9.9-	10.9	10.4	11.9
Mean viable litter size at birth	11.0	11.7	9.7-	10.4	9.9	10.9	9.9	11.9
Pups with external abnormalities	1	0	5	0	0	0	0	0
Live birth index (%)	97.3	100	99.2	100	100.0+	100	95.2	100
Day 4 survival index	98.8	95.4	94.5-	98.2	95.3-	97.5	98.3	97.3
Day 7 survival index	99.1	100	100.0	100	99.0	98.8	99.5	98.6
Day 14 survival index	95.2	97.0	89.9-	99.0	99.0+	98.8	99.1+	95.5
Day 21 survival index	94.8	96.0	87.8-	99.0	97.5	98.2	99.1+	95.0

-significantly decreased
+significantly increased

Table B.6.6.1/04-13: pup body weights of F1 and F2 pups during lactation (group mean values)

Dietary concentration (ppm)	No. of litters		Body weight (g) at Day			
			1	4	14	21
			F1 Generation			
0 (Control)	29	mean	5.5	7.8	21.8	31.2
		SD	0.6	0.9	3.2	5.7
100	26	mean	5.8+	8.5	20.6	30.0
		SD	0.7	1.3	3.5	6.0
1000	28	mean	6.1+	8.6+	23.3	36.3+
		SD	1.1	1.4	3.0	6.0
10000	30	mean	6.0+	8.6	22.6	32.7
		SD	0.7	1.1	2.4	4.4
			F2 Generation			
0 (Control)	26	mean	5.4	7.4	22.9	35.0
		SD	0.6	1.2	2.2	3.8
100	27	mean	5.7+	8.1+	23.1	33.8
		SD	0.7	1.2	2.6	4.4
1000	22	mean	5.9+	8.4+	22.7	33.3
		SD	0.6	1.2	3.0	6.1
10000	28	mean	5.6+	7.6	21.6	32.0
		SD	0.4	0.7	3.0	4.2

SD standard deviation
+ significant higher than control

PATHOLOGY

Necropsy

F0 generation:

The gross pathological lesions seen were consolidated lungs with ecchymoses, chronic liver changes, kidneys with cysts and dilated pelvis, and hypoplastic testes (1 in the control group, 2 in the mid-dose and 1 in the high-dose group). The incidence was low and did not appear to be compound or dose related.

F1 generation:
The gross pathological lesions seen were consolidated and collapsed lungs with emphysema, hydronephrotic kidneys, and unilateral hypoplastic testes. The lesions observed were few and appeared to be incidentally. A single incidence of unilateral testicular hypoplasia was observed in each of the three treatment groups, hydronephrosis was seen in two animals in the high dose group.

F1 pups:
A higher incidence of emaciated pups was recorded for the mid and high dose groups compared to controls. A low incidence of minor developmental abnormalities like kinked tail, rudimentary tail, kidney hydro-nephrosis and dilated pelvis occurred without dose-response relation.

Table B.6.6.1/04-14: Summary of necropsy findings- parents (P0)

PARTICULARS	Group No.	G1		G2		G3		G4	
	Dose (ppm)	0		100		1000		10000	
	Sex	M	F	M	F	M	F	M	F
1. Total animals in the group		30	30	30	30	30	30	30	30
2. No. dead during treatment		0	0	0	2	0	0	0	1
3. No. of moribund sacrifice		0	0	0	0	0	0	0	0
4. No. finally sacrificed		30	30	30	28	30	30	30	29
5. No. examined for gross pathology		30	30	30	30	30	30	30	30
6. No. showing gross pathology		4	2	0	2	5	0	1	1
a. External pathology		0	0	0	0	0	0	0	0
b. Visceral organ pathology		4	2	0	2	5	0	1	1
i) Pituitary congestion		0	0	0	1	0	0	0	0
ii) Lungs									
a. Petechiae		1	0	0	0	0	0	0	0
b. Suppurative pneumonia		0	0	0	1	0	0	0	0
c. Ecchymoses		0	1	0	0	2	0	0	0
d. Enlarged		0	1	0	0	0	0	0	0
e. Focal consolidation		0	1	0	0	0	0	0	0
iii) Liver									
a. Mottled		0	1	0	1	0	0	0	0
b. Chronic change		1	0	0	0	0	0	0	0
iv) Kidneys									
a. Cyst		1	0	0	0	0	0	0	0
b. Pelvis dilated		0	0	0	0	1	0	0	0
v) Testes									
Hypoplasia		1	NA	0	NA	2	NA	1	NA
vi) Autolysis		0	0	0	1	0	0	0	1

Table B.6.6.1/04-15: Summary of necropsy findings- parents (P1)

PARTICULARS	Group No.	G1		G2		G3		G4	
	Dose (ppm)	0		100		1000		10000	
	Sex	M	F	M	F	M	F	M	F
1. Total no.of animals in the group		30	30	30	30	30	30	30	30
2. No. dead during treatment		0	0	0	1	0	0	0	0
3. No. of moribund sacrifice		0	0	0	0	0	0	0	0
4. No. finally sacrificed		30	30	30	29	30	30	30	30
5. No. examined for gross pathology		30	30	30	30	30	30	30	30
6. No. showing gross pathology		0	0	1	1	2	1	5	1
a. EXTERNAL PATHOLOGY Emaciation		0	0	0	0	0	1	0	0
b. VISCERAL ORGAN PATHOLOGY		0	0	1	1	2	0	5	1
I. LUNGS i) Focal Consolidation		0	0	0	0	0	0	2	0
ii) Collapse		0	0	0	0	0	0	1	0
iii) Emphysema		0	0	0	0	0	0	2	0
iv) Ecchymoses		0	0	0	0	0	0	1	0
II. LIVER White foci.		0	0	0	0	0	0	0	1
III. KIDNEY i) Hydronephrosis		0	0	0	0	0	0	2	0
ii) Pelvis dilated		0	0	0	0	1	0	0	0
IV. TESTES - Unilateral Hypoplasia		0	NA	1	NA	1	NA	1	NA
V. AUTOLYSIS		0	0	0	1	0	0	0	0

Table B.6.6.1/04-16: Summary of necropsy findings F1 pups

	Dietary concentration (ppm)							
	0		**100**		**1000**		**10000**	
Sex	M	F	M	F	M	F	M	F
Animals examined	125	135	102	84	94	115	123	119
Cannibalism/Missing	5	7	15	9	7	10	2	5
General Observations								
Emaciation	0	2	0	0	3	2	2	4
Autolysis	5	7	8	8	1	0	8	7
Developmental malformations								
External malformations								
Kinked tail	0	0	2	3	0	0	0	0
Rudimentary tail	0	1	0	0	0	0	0	0
Visceral organ pathology								
Kidney-hydronephrosis	0	0	0	0	2	0	1	1
Kidney – pelvis dilated	1	1	0	0	0	0	0	0

F2 pups:
A higher incidence of emaciation has been observed in pups of high dose group. Occasional not treatment and dose related incidence of hydronephrosis and dilated pelvis in kidney have been recorded.

Table B.6.6.1/04-17: Summary of necropsy findings F2 pups

	Dietary concentration (ppm)							
	0		**100**		**1000**		**10000**	
Sex	M	F	M	F	M	F	M	F
Animals examined	167	138	133	148	134	105	161	173
Cannibalism/Missing	10	11	5	3	5	2	6	12
General Observations								
Emaciation	0	0	0	0	1	0	5	3
Autolysis	0	1	0	0	0	1	2	0
Developmental malformations								
External malformations	0	0	0	0	0	0	0	0
Visceral organ pathology								
Kidney-hydronephrosis	0	2	0	0	0	0	0	0
Kidney – pelvis dilated	0	0	0	0	0	0	1	0

HISTOPATHOLOGY

F0 generation:

Reproductive organs showing gross pathological changes were recorded as outlined in the following: testes from one control animal, two mid dose and one high dose animal. The control and high dose animals showed degenerative changes in the seminiferous tubules while the mid dose group did not show this finding. These changes appeared to be incidental and not compound related.

Table B.6.6.1/04-18: Summary of histopathological findings – F0 males

Observations	**Dietary concentration (ppm)**			
	0	**100**	**1000**	**10000**
No. of tissues evaluated	1	0	2	1
Testes				
Degenerative changes – seminiferous tubules	1	0	0	1

F1 generation:

Reproductive organs showing gross pathological changes were recorded as outlined in the following: testes from one animal in each of the three treatment groups; the testes in the low and mid dose groups showed unilateral degenerative changes and giant cell formation in the seminiferous tubules and focal chronic inflammation. The testes in the high dose were normal though unequal in size. The changes appeared to be incidental and not compound related.

Table B.6.6.1/04-19: Summary of histopathological findings – F1 males

Observations	**Dietary concentration (ppm)**			
	0	**100**	**1000**	**10000**
No. of tissues evaluated	0	1	1	1
Testes				
Degenerative changes - seminiferous tubules	0	1	1	0
Giant cell formation - seminiferous tubules	0	1	1	0
Inflammation – chronic (focal)	0	1	1	0

CONCLUSION BY STUDY AUTHOR

The two generation reproduction study conducted on Glyphosate Technical at dietary dose levels of 0, 100, 1000 and 10000 ppm has shown that at the dosages tested up to the weaning of F2 litters the test compound has no major effects on general health, growth of parents; gestation/lactation period, body weight and feed consumption, gross necropsy findings of pups and parents. The test compound did not cause any treatment or dose related consistent changes in parental mortality, parturition performance, mean litter size, pup weight and

male and female fertility index. Thus, the study has indicated that at the tested doses and conditions adopted Glyphosate Technical has no toxicity effects on reproductive performance of Wistar rats. As per this study NOAEL is more than 100 ppm in diet or 7.7 mg of Glyphosate Technical per kilogram body weight.

The oral administration of glyphosate to rats by dietary admixture at a maximum dose level of 10000 ppm for two successive generations of Wistar rats resulted in no parental toxicity. The NOAEL for reproduction is considered to be 10000 ppm, since the reproductive performance was not affected in a dose-related manner. The NOAEL for offspring is 10000 ppm since no treatment-related effects on offspring were observed.

Assessment and conclusion by applicant:
The highest dose of 10000 ppm is considered to be the NOAEL for parental, reproductive and offspring toxicity. This dietary level would correspond to a mean daily compound intake of 700-800 mg/kg bw/day.

Assessment and conclusion by RMS:
In this study groups of 30 males and 30 females Wistar (Random bred) rats were fed diets containing glyphosate technical at concentrations of 0, 100, 1000, and 10000 ppm for two successive generations. No treatment-related effects were observed. However, it could be noted that the study was limited (effect dose lacking, no sperm analyses, sexual offspring development not investigated, limited histopathology).

The NOAELs in previous evaluation RAR remains.

NOAEL for parental, reproductive and offspring toxicity was set at 10000 ppm (highest tested dose). This dietary level would correspond to a mean daily compound intake of 700-800 mg/kg bw/d. [The mean daily intake was not reported for all dietary levels, but for the low level of 100 ppm a corresponding average value of 7.7 mg/kg bw/d was given in the original report].

The study is supplementary only (effect dose lacking, limited parameters investigated in study). The study is conducted in accordance with GLP. The study was checked for compliance with OECD TG 416 (2001) and following deviations were observed:
(i) the highest dose level was too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering
(ii) details of achieved concentrations not presented
(iii) Parental animals were dosed at least 8 weeks before the mating period (the guideline recommend that dosing shall be continued for at least 10 weeks before the mating period)
(iv) estrous cycle monitoring not performed
(v) pre-coital interval not recorded
(vi) data for gestation length not presented
(vii) sperm analysis not performed
(viii) monitoring of physical and sexual offspring development not performed
(ix) organ weights not determined
(x) a quantitative evaluation of primordial follicles not conducted
(xi) no histopathology (only for organs found abnormal in the macroscopical investigation)

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- sperm morphology
- sperm motility

- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- epididymis histopathology
- epididymis weight
- liver weight
- ovary histopathology
- ovary weight
- prostate histopathology
- prostate weight
- seminal vesicles histopathology
- seminal vesicles weight
- testis histopathology (except for animals with grosspathological findings in testis)
- testis weight
- uterus histopathology
- uterus weight
- vagina histopathology

Sensitive to but not diagnostic of EATS:

- number of corpora lutea
- pre- and post-implantation loss
- time to mating
- adrenal weight
- pituitary weight
- gestation length

B.6.6.1/07 / B.6.6.1/08

Data point	CA 5.6.1/007
Report author	[redacted]. *et al.*
Report year	1992
Report title	The Effect of Dietary Administration of Glyphosate on Reproductive Function of Two Generations in the Rat (Vol. 1)
Report No.	[redacted] 47/911129
Document No.	Not reported
Guidelines followed in study	OECD 416 (1983), US-EPA FIFRA 83-4 (1982)
Deviations from current test guideline	• no determination of pre-mating oestrous cycles • no sperm analysis • some organ weights of parental animals missing • organ weights of offspring missing Comments by RMS: The following deviations were observed in addition: • pre- and post-implantation loss were not reported Following organs of parental animals were not weighed: uterus, spleen and thyroid. Following organs of pups were not weighed: brain, spleen and thymus
Previous evaluation	Yes, evaluated and accepted in DAR (1998) and RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Yes

Data point	CA 5.6.1/008
Report author	[redacted] *et al.*
Report year	1992
Report title	The Effect of Dietary Administration of Glyphosate on Reproductive Function of Two Generations in the Rat (Vol. II)
Report No.	[redacted] 47/911129
Document No.	Not reported
Guidelines followed in study	OECD 416 (1983), US-EPA FIFRA 83-4 (1982)
Deviations from current test guideline	See above
Previous evaluation	Yes, evaluated and accepted in DAR (1998) and RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material:	Glyphosate technical
Lot/Batch:	206-JaK-119-1 Stability of test compound: Stable during the treatment period.
Purity/Radiochemical purity:	Purity: 99.2 % (w/w)
Vehicle:	Plain diet
Animals:	Species: Rat Strain: Sprague-Dawley Crl:CD (SD) BR VAF/Plus Age: Approximately 6 weeks Sex: Males and females Weight at dosing: Males: 143 – 201 g; females: 106 – 175 g

Acclimation period: At least 15 days
Diet/Food: Biosure Laboratory Animal Diet No.2, *ad libitum*
Water: Tap water, *ad libitum*
Housing: During pre-mating periods, animals were housed in groups of four in metal cages with wire mesh front, floor and top. During the first week of F1A and contingency animals of F2B animals were housed in plastic cages. During mating animals were housed on an 1:1 basis in plastic cages where females stayed after mating for breeding. Males were re-housed in former metal cages.
Environmental conditions:
Temperature: 23 ± 4 °C
Humidity: 46% ± 24 %
Air changes: not reported
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In a two-generation reproduction study groups of 28 Sprague-Dawley rats per sex of the F0 generation received daily dietary doses of 0, 1000, 3000 and 10000 ppm glyphosate technical. The dose levels were chosen based on results of a previously conducted study (Report No. [redacted] 42/90619) in which treatment at 3000, 10000 and 30000 ppm, was associated with signs of maternal toxicity, whilst effects on development of the offspring to 6 weeks of age was not affected up to 10000 ppm.

After at least 70 days of treatment pairing of animals within each dose group was undertaken on a 1:1 basis to produce the F1 litters. At Day 21 *post partum* of offspring from the F0 mating phase, groups of 24 male and 24 female offspring from each dose group were selected to form the F1A generation. The remaining pups were sacrificed. Approximately 10 days following the weaning of all F1A pups, F0 males and females were re-mated.

At Day 21 *post partum* all F1B pups were sacrificed. F0 males and females were terminated shortly after weaning of F1B pups.

The selected F1A animals were dosed from approximately Week 4 of age for at least 84 days and then mated on a 1:1 basis (sibling pairings were avoided). On Day 4 *post partum* F2A litters were standardised to 8 pups per litter (4 males and 4 females where possible). The remaining pups were sacrificed. On or shortly after Day 21 *post partum* all F2A pups were sacrificed. Approximately 10 days following the weaning of all F2A pups, F1 males and females were re-mated. On Day 4 *post partum* F2B litters were standardised to 8 pups per litter. The remaining pups were sacrificed. On or shortly after Day 21 *post partum* all F2B pups were sacrificed. F1 males and females were terminated shortly after weaning of F2B pups.

Diet preparation and analyses
For the weekly preparation of diet mixtures a known amount of the test substance was mixed with a small amount of basal diet. This pre-mix was then added to larger amount of basal diet and blended for further 7 minutes in a rotary double-cone-blender. Fresh diet was prepared weekly and fed for no more than 15 days from the date of preparation.

The stability and homogeneity of the test material in diet were determined. Dietary admixtures were analysed for achieved concentration throughout the study.

Clinical observations
A check for clinical signs or ill health was made once daily and recorded daily for the first week of treatment and on a weekly basis thereafter. Rats showing marked signs of ill health or reaction to treatment were killed and subjected to necropsy.

Body weight
Individual body weights were recorded at the start of each generation (F0: Week 6 of age; F1A: Week 4 of age) and subsequent at weekly intervals. Females were weighed daily during mating and continued until parturition. Weights were reported for Days 0, 7, 14, 17 and 20 of pregnancy. Females with live litters were weighed on Days 0, 7, 14 and 21 *post partum*.

Food consumption and compound intake
Food consumption was recorded on a weekly basis from allocation throughout the first pre-mating phase of each generation. During this period food conversion ratios and achieved intake (mg/kg bw/day) were calculated.

Water consumption
Water intake was observed daily during the initial and final two weeks of the first pre-mating period for each generation and from allocation for the F0 generation.

Reproduction parameters
Vaginal smears were taken daily during the 20–day mating period to examine the oestrus cycle and median pre-coital time. Additionally, date of mating and duration of gestation was recorded.

Litter data
The number of offspring born and the number of offspring alive were recorded daily. Pups were weighed on Days 0 and 4 and all litters containing more than eight pups were culled to eight on Day 4 *post partum*, retaining, where possible, ideally 4 pups per sex. The remaining pups were also weighed on Days 8, 12, 16 and 21 *post partum*. Dead and culled young were subjected to necropsy.

Sacrifice and pathology
All adult animals were subjected to a full external and internal examination, and any macroscopic abnormalities were recorded.

The following organs were weighed of adults: adrenals, brain, heart, kidneys, liver, lungs, ovaries, prostrate (with seminal vesicles and coagulating gland), testes (with epididymides), thymus.

The following tissues were preserved from all adults: adrenals, aorta, bone (femur and joint), bone marrow (sternum), brain, cranial vault (for lachrymal glands, teeth, nasal turbinates, inner ear), caecum, colon, duodenum, eyes, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes (cervical/mesenteric), mammary gland, macroscopically abnormal tissues, oesophagus, ovaries*, pancreas, pituitary*, prostate with seminal vesicles (with coagulating gland)*, rectum, salivary gland, sciatic nerve, skeletal muscle, skin, spinal column (vertebral column), spleen, stomach, testes (with epididymides)*, thymus, thyroids (with parathyroids), tongue, trachea (with larynx and pharynx), urinary bladder, uterus (with cervix)* and vagina*.

Histology of the reproductive tract was restricted to adults of the control and high-dose group and any apparently infertile animals at the lower dietary concentrations and confined to tissues marked with an asterisk (*).

Statistics
Two tailed significance tests were performed on adult parameters (water consumption, food consumption, body weight, organ weights) and litter data. Evaluation of other parameters were found not to be useful. Significances at 1% and 5% were reported.

RESULTS

ANALYSIS OF DOSE FORMULATIONS
Stability analyses indicated that the dose preparations at nominal concentrations of 500 and 30000 ppm were stable for up to 18 days during storage under animal room conditions.

Analyses for homogeneity at nominal concentrations of 500 and 30000 ppm indicated that the dose preparations were homogeneous.

Analyses for achieved concentration performed at 4-5 weekly intervals demonstrated that the prepared dietary admixture concentrations given to the animals were within ± 15% of the nominal concentration in all groups.

TEST COMPOUND INTAKE
The group mean intakes of glyphosate are summarised in the tables below.

Table B.6.6.1/05-01: Group mean achieved intakes of glyphosate - F0 generation

Group	Dietary concentration	Mean intakes (Week 1 - 10)	
	(ppm)	(mg/kg bw/day)	
		Males	Females
Control	0	0	0
Low	1000	66.4	75.3
Intermediate	3000	196.8	226.0
High	10000	668.1	752.3

Table B.6.6.1/05-02: Group mean achieved intakes of glyphosate - F1 generation

Group	Dietary concentration	Mean intakes (Week 5 - 16)	
	(ppm)	(mg/kg bw/day)	
		Males	Females
Control	0	0	0
Low	1000	76.1	82.1
Intermediate	3000	230.2	244.9
High	10000	771.3	841.1

MORTALITY
There were no test substance related mortalities.

Four unscheduled deaths occurred during each generation.

In the F0 generation one female of the low dose group and one male of the high dose group were killed for humane reasons during Week 15 and 23, respectively. The female exhibited pilo-erection and thin appearance and the necropsy noted thickened forestomach, invaginated stomach and abnormal contents in the gastro-intestinal tract. The male was unable to use hind limbs, exhibiting aberrations of brain and spinal cord at necropsy. Another male of the high dose group died during Week 3, with effects on pancreas (pale, oedematous) and liver (swollen, pitted) noted at necropsy. One control male was sacrificed during Week 16 following poor condition, however, the aetiology of the signs was not established.

In the F1 generation one female of the low dose group was killed following a procedural error. In the mid dose group one male died during Week 34 but autolytic changes precluded a valid necropsy. One male of the high dose group died during Week 23. *Post mortem* examination did not reveal the specific cause of death. One group 4 female of high dose was sacrificed during Week 23 following signs of pale extremities, weakness, unsteadiness, soft faeces, yellow staining in the urogenital region and weight loss. *Post mortem* examination revealed minimal gastro-intestinal contents, enlarged adrenals and accentuated lobular marking on the liver.

None of these mortalities are considered to be obviously related to treatment.

CLINICAL OBSERVATIONS
No specific signs of reaction to treatment were observed in adults of either generation. General signs including skin ulceration, hair loss/scabbing, brown staining of the coat, red/brown eye discharge, dental anomalies and soft faeces were observed in occasional animals from both generations.

BODY WEIGHT
No adverse effect of body weight change was evident for treated animals in comparison to controls for both generations.

However, absolute mean body weights in high dose F1 males were slightly lower as compared to control (at nominal Week 4). In addition, it was noted that during the first mate of each generation, body weight gains during the initial stages of pregnancy tended to be slightly lower than controls at all dietary levels. Since no consistent dose-response was apparent these effects cannot conclusively be attributed to treatment.

Table B.6.6.1/05-03: Group mean body weights (g) - F0 generation

Week	Males				Females			
	Dietary concentration (ppm)				Dietary concentration (ppm)			
	0	1000	3000	10000	0	1000	3000	10000
0	233	233	236	235	173	171	171	170
5	452	454	459	449	272	275	270	270
9	535	539	546	530	304	308	304	304
10[1]	550	556	565	546	311	314	312	312
15	595	603	615	591	352	347	360	351
19	641	650	664	638	349	355	349	347
20[2]	650	661	674	649	353	360	353	351
25	664	672	692	664	410	400	408	403
28	704	710	732	702	381	381	377	370

[1] First mating commenced
[2] Second mating commenced

Table B.6.6.1/05-04: Group mean body weights (g) - F1 generation

Week	Males				Females			
	Dietary concentration (ppm)				Dietary concentration (ppm)			
	0	1000	3000	10000	0	1000	3000	10000
4	108	104	106	100 (7%)	95	93	98	93
10	425	429	423	412 (3%)	266	265	270	267
15	526	542	521	512 (3%)	316	313	325	319
16[1]	540	555	538	528 (2%)	324	321	335	326
21	605	619	600	591 (2%)	373	370	373	381
26	659	677	659	649 (1.5%)	367	368	380	370
27[2]	673	692	671	663 (1%)	374	372	390	376
32	710	727	709	699 (1.5%)	413	424	436	424
37	762	781	746	742 (3%)	415	410	424	417

[1] First mating commenced
[2] Second mating commenced

FOOD AND WATER CONSUMPTION

Apart from a slightly higher but not statistically significant food consumption of high dose F1 females during the second half of the pre-mating period, there were no marked intergroup differences in food consumption of males or females. Across both generations, there were no clear or consistent adverse effects of treatment on the food conversion ratio, indicating that treatment with glyphosate does not overtly affect the efficiency of food utilisation into body weight of either sex at dietary concentrations of up to 10000 ppm.

Apart from a slight increase among high dose F1 females (attaining statistical significance in Week 16), no overt intergroup differences in water intake for treated males and females from the F0 or F1 generations when compared to their concurrent controls.

Table B.6.6.1/05-05: Group mean weekly food consumption (g/rat/week) - F0 generation

Week	Males				Females			
	Dietary concentration (ppm)				Dietary concentration (ppm)			
	0	1000	3000	10000	0	1000	3000	10000
1	188	191	190	185	136	134	134	129
5	188	191	191	193	136	136	135	135
10	184	185	186	184	125	128	129	128

Table B.6.6.1/05-06: Group mean weekly food consumption (g/rat/week) - F1 generation

Week	Males				Females			
	Dietary concentration (ppm)				Dietary concentration (ppm)			
	0	1000	3000	10000	0	1000	3000	10000
5	133	125	134	124	108	106	111	108
10	190	195	189	187	140	139	144	147
13	190	193	191	191	135	137	140	143
16	181	184	184	180	128	128	131	132

Table B.6.6.1/05-07: Water consumption- group mean weekly values (g/rat/week) F0 generation

Group: 1 2 3 4

Compound: Control Glyphosate

Dietary inclusion (ppm): - 1000 3000 10000

Week	Group							
	1♂	2♂	3♂	4♂	1♀	2♀	3♀	4♀
-1	190.9	190.0	195.5	190.5	169.5	167.5	164.9	168.9
1	215.9	215.9	222.2	217.6	179.5	186.1	179.3	183.3
2	237.8	235.1	244.8	235.6	191.0	199.7	200.0	199.1
9	242.5	244.4	253.1	237.7	201.9	226.5	201.4	197.0
10	233.1	236.0	249.1	228.5	197.8	218.8	200.3	187.1
Cumulative 1 - 10	929.3	931.4	969.1	918.4	770.3	831.0	781.1	766.5
% of control	-	100	104	99	-	108	101	100

Statistical analysis

Analysis of variance followed by intergroup comparison with the controls (Williams' test) not significant for all weeks (P>0.05)

Table B.6.6.1/05-08: Water consumption- group mean weekly values (g/rat/week) F1 generation

Group:	1	2	3	4
Compound:	Control		Glyphosate	
Dietary inclusion (ppm):	-	1000	3000	10000

Week	Group							
	1♂	2♂	3♂	4♂	1♀	2♀	3♀	4♀
5	159.3	150.4	157.3	156.2	143.2	141.7	141.6	152.9
6	198.9	200.4	199.6	201.2	175.8	176.6	178.4	192.4
15	230.5	249.2	237.8	239.3	184.3	188.6	188.2	204.6
16	227.0	242.3	236.3	239.8	175.7	182.9	182.3	206.3**
Cumulative 5 - 16	815.8	842.3	831.0	836.5	679.0	689.8	690.6	756.2
% of control	-	103	102	103	-	102	102	111

Statistical analysis

Analysis of variance followed by intergroup comparison with the controls (Williams' test), significant at; ** P<0.01

REPRODUCTIVE PARAMETERS

There were no treatment-related effects on mating performance, fertility and gestation length for both F0 and F1 generation animals.

Table B.6.6.1/05-09: Summary of adult performance - F0 generation

Parameter	**Dietary concentration (ppm)**			
	0	**1000**	**3000**	**10000**
Males				
Initial group size	28	28	28	28
Died (pre-mating 1)	-	-	-	1
Sacrificed (pre-mating 2)	1	-	-	-
Sacrificed (post-mating 2)	-	-	-	1
Failed to induce pregnancy in either female partner	-	-	-	1
Females				
First mating				
Initial group size/mated	28	27	28	28
Median pre-coital time (days)	2.0	3.0	2.5	3.0
Mean duration of pregnancy (days)	22.0	22.2	22.0	22.1
No young born	-	-	1	2
Non-pregnant[1]	1	2	-	1
Pregnancy rate (%)	96.4	92.9	96.4	89.3
Sacrified *post partum*	-	1	-	-
Rearing young to weaning	27	25	27	25
Second mating				
Initial group size/mated	28	27	28	28
Median pre-coital time (days)	2.5	3.0	3.0	3.0
Mean duration of pregnancy (days)	22.1	21.9	21.9	22.0

Parameter	**Dietary concentration (ppm)**			
	0	**1000**	**3000**	**10000**
No young born	2	2	-	1
Non-pregnant[1]	1	2	-	1
Pregnancy rate (%)	89.3	85.2	100.0	92.9
Total litter loss *post partum*	1	1	-	1
Rearing young to weaning	24	22	28	25
Failed to mate with both male partners	1	2	-	1

[1] No implantation sites detected by Salewski technique at termination following second mating

Table B.6.6.1/05-10: Summary of adult performance – F1 generation

Parameter	**Dietary concentration (ppm)**			
	0	**1000**	**3000**	**10000**
Males				
Initial group size	24	24	24	24
Died (pre-mating 2)	-	-	-	11
Died (post-mating 2)	-	-	1	-
Failed to induce pregnancy in either female partner	3	1	3	31
Females				
First mating				
Initial group size/mated	24	24	24	24
Median pre-coital time (days)	3.0	3.0	6.0	3.0
Mean duration of pregnancy (days)	22.2	22.0	22.1	22.1
No young born	1	3	3	2
Non-pregnant[1]	3	3	7	3
Pregnancy rate (%)	83.3	75.0	58.3	79.2
Total litter loss *post partum*	1	1	-	-
Rearing young to weaning	19	17	14	19
Second mating				
Sacrificed prior to mating	-	1[3]	-	1
Group size of females for mating	24	23	24	23
Median pre-coital time (days)	3.5	2.5	4.5	4.0
Mean duration of pregnancy (days)	22.3	22.1	22.2	22.3
No young born	5	2	1	3
Non-pregnant[2]	3	3	7	3
Pregnancy rate (%)	66.7	78.3	66.7	73.9
Total litter loss *post partum*	-	1	1	-
Rearing young to weaning	16	17	15	17
Failed to mate with both male partners	3	3	7	3

[1] Failed to mate with partner at first mating, included in failed to mate
[2] No implantation sites detected by Salewski technique at termination following second mating
[3] Female mistakenly placed in male cage after first mating and became pregnant

LITTER DATA
Size and Viability
No overt differences in litter viability were detected.

In the high-dose group total litter size at birth was consistently, but not significantly, lower than controls across all four matings and remained lower than controls at Day 4 in three of the four matings. Since the mean litter size

at birth within each mating, was not always the lowest litter size recorded, this finding could not be clearly attributed to treatment.

Growth and Development
No adverse effects on mean offspring body weights, body weight change or development were detected for male and female offspring in comparison to their controls.

Table B.6.6.1/05-11: Litter data F0 generation

Parameter	Dietary concentration (ppm)			
	0	**1000**	**3000**	**10000**
First Mating				
Mean litter size				
at birth (live pups)	15.0	14.0	14.3	14.0
at day 4 *post partum* (pre-cull)	14.5	13.3	14.0	13.7
at day 8 *post partum*	7.9	7.6	7.9	7.8
at day 21 *post partum*	7.9	7.6	7.9	7.8
Mean pup weight (g)				
at birth	6.2	6.2	6.2	6.4
at day 4 *post partum* (pre-cull)	10.0	9.8	10.1	10.3
at day 8 *post partum*	19.6	18.5	19.3	19.3
at day 21 *post partum*	60.7	56.6* (7%)	57.6* (5%)	56.0** (8%)
Percentage male pups (%)				
at birth	49.9	50.8	57.7	54.4
At day 21 *post partum*	50.9	49.4	52.2	51.8
Second Mating				
Mean litter size				
at birth (live pups)	15.0	15.7	14.6	15.1
at day 4 *post partum* (pre-cull)	14.7	15.2	14.4	14.9
at day 8 *post partum*	7.9	7.8	7.6	7.8
at day 21 *post partum*	7.9	7.7	7.5	7.8
Mean pup weight (g)				
at birth	6.2	6.3	6.6	6.4
at day 4 *post partum* (pre-cull)	10.0	9.8	10.4	10.4
at day 8 *post partum*	19.6	19.4	20.1	20.5
at day 21 *post partum*	60.9	59.2	60.4	59.0
Percentage male pups (%)				
at birth	52.3	54.5	53.7	47.8
At day 21 *post partum*	49.6	52.0	50.0	49.0

*/** significant at P<0.05/0.01; Kruskal-Wallis 'H' statistic followed by intergroup comparison with the controls (Shirley's test)

Table B.6.6.1/05-12: Litter data F1 generation

Parameter	Dietary concentration (ppm)			
	0	**1000**	**3000**	**10000**
First Mating				
Mean litter size				
at birth (live pups)	13.8	12.4	14.1	13.2
at day 4 *post partum* (pre-cull)	13.6	12.2	13.9	12.9
at day 8 *post partum*	7.8	7.6	8.0	7.6
at day 21 *post partum*	7.8	7.6	8.0	7.6
Mean pup weight (g)				
at birth	6.4	6.5	6.4	6.7
at day 4 *post partum* (pre-cull)	10.1	10.7	10.2	11.1
at day 8 *post partum*	19.2	20.0	19.0	20.8

Parameter	Dietary concentration (ppm)			
	0	**1000**	**3000**	**10000**
at day 21 *post partum*	57.2	56.6	56.4	58.7
Percentage male pups (%)				
at birth	53.0	53.0	44.9	51.3
At day 21 *post partum*	47.8	51.2	48.2	51.8
Second Mating				
Mean litter size				
at birth (live pups)	14.7	12.8	15.0	13.1
at day 4 *post partum* (pre-cull)	13.9	12.6	14.7	13.0
at day 8 *post partum*	7.9	7.5	7.8	7.5
at day 21 *post partum*	7.9	7.4	7.4	7.4
Mean pup weight (g)				
at birth	6.4	6.7	6.4	6.7
at day 4 *post partum* (pre-cull)	10.1	11.0	9.9	11.3
at day 8 *post partum*	19.1	20.6	18.3	21.2
at day 21 *post partum*	60.4	62.4	59.5	60.4
Percentage male pups (%)				
at birth	57.9	51.6	51.6	56.1
At day 21 *post partum*	50.4	51.0	54.2	55.2

Table B.6.6.1/05-13: Mean sexual maturation landmarks F1 generation

Parameter	Dietary concentration (ppm)			
	0	**1000**	**3000**	**10000**
Balanopreputial separation (day of age)	41.8[1]	42.5	42.2	42.6
Vaginal opening (day of age)	33.7	33.5	33.2	33.9

[1] In the study report, this value was given incorrectly as 39.9. This was already noted in RAR 2015. Recalculation resulted in the given value

Clinical signs
No clinically observable signs of toxicity were observed for offspring from treated animals.

PATHOLOGY
Necropsy
There were no toxicologically significant macroscopic abnormalities detected in the F0 and F1 animals, or offspring.

Organ weights
There were no overt or statistically significant treatment-related changes in any organ weights analysed in either generation.

Histopathology
No treatment-related changes in tissues associated with the reproductive tract were detected in the F0 or F1 generation animals.

Examination of two previously identified target organs, the parotid and submaxillary salivary glands, was initially performed only in the control and high-dose groups. Due to effects seen in the parotid gland, examination was extended to the remaining treatment groups. For the submaxillary gland, examination was extended to only the F0 and F1 females in the low- and mid dose group. The findings are summarised in the table below.

Table B.6.6.1/05-14: Incidence of salivary gland findings

Observation	Dietary concentration (ppm)							
	Males				Females			
	0	1000	3000	10000	0	1000	3000	10000
F0 Generation								
Animals examined	27	28	28	26	28	27	28	28
Hypertrophy of acinar cells with prominent granular cytoplasm (minimal)								
parotid	2	2	3	12	0	2	5	17
submaxillary	0	-	-	0	0	1	4	14
F1 Generation								
Animals examined	24	24	23	23	24	23	24	23
Hypertrophy of acinar cells with prominent granular cytoplasm (minimal)								
parotid	1	0	4	11	0	0	4	9
submaxillary	0	-	-	0	0	0	0	3

- = not examined

Treatment-related minimal changes were apparent in the parotid salivary gland of both F0 and F1 males and females in the mid- and high-dose groups, and the submaxillary salivary gland of the F0 females in the mid- and high-dose groups and F1 females in the high-dose group. This finding occurred with a dose-related incidence in the F0 as well as in the F1 adults.

CONCLUSIONS BY STUDY AUTHOR

The oral administration of glyphosate technical to rats by dietary admixture at a maximum dose level of 10000 ppm for two successive generations resulted in minimal effects consisting of increased food and water consumption of F1 females, possibly reduced body weights of F1 males and minimal histological changes in the target organ (salivary glands) in F0 and F1 adults at 10000 ppm. The only findings associated with treatment at 3000 ppm were minimal histopathological changes of the salivary glands in F0 and F1 adults. No effects were apparent at 1000 ppm. With the exception of minimal changes to the target organ, it is considered that the non-toxic effect level for non-reproductive and reproductive events was 3000 ppm.

Assessment and conclusion by applicant:

No evidence of reproductive effects was observed. The only findings associated with treatment at 3000 ppm were minimal histopathological changes of the salivary glands in F0 and F1 adults considered to be an adaptive response due to oral irritation from the ingestion of glyphosate, an organic acid, in the diet and is not considered to be adverse (for mechanistical studies on salivary gland effects see also section 5.8.2). No effects were apparent at 1000 ppm. Thus, the parental, reproductive and offspring NOAELs are considered to be 10000 ppm, corresponding to 668 and 752 mg/kg bw/day in males and females, respectively.

Assessment and conclusion by RMS:

Dietary administration of glyphosate technical to two successive generations of Sprague-Dawley Crl:CD (SD) BR VAF/Plus rats at concentrations of 0, 1000, 3000, and 10000 ppm did not affect the fertility and reproductive performance of each generation of parental animals, and there was no indication of a teratogenic effect of the test substance at any of these concentrations. In previous RAR 2015 the NOAEL for parental and offspring toxicity was set at 3000 ppm: "*The NOAEL for parental and offspring toxicity is calculated to be 3000 ppm (197 mg/kg bw/d) based on increased food and water consumption in F1 females, lower body weight of F1 males and an increased incidence of cellular alteration of the parotid (males and females) and submaxillary (females) salivary gland in both F0 and F1 adults at 10000 ppm (Evaluation in 2001 confirmed).*

For the renewal of active substance, the NOAEL for parental toxicity is proposed to be changed from 3000 ppm to 1000 ppm.

Treatment was associated with following findings:

- An increase (17%) in water consumption of F1 generation females at 10000 ppm
- A slight increase (3%) in food intake of F1 10000 ppm females during the latter stage of the first pre-mating Period
- A slightly lower (1-7%) absolute mean bodyweight of adult F1 10000 ppm males apparent at selection, although overall weight gain from this point was comparable with controls
- Histopathological changes to the parotid and submaxillary salivary glands, manifested as minimal hypertrophy of acinar cells with prominent granular cytoplasm observed at ≥3000 ppm. The findings in the parotid gland were observed in male and female animals of both generations at 3000 ppm (F0 males 3/28, F0 females 5/28, F1 males 4/23, F1 females 4/24) and 10000 ppm (F0 males 12/26, F0 females 17/28, F1 males 11/23, F1 females 9/23). The findings in the submaxillary gland were observed in F0 females at 3000 ppm (F0 females 4/28), and in F0 and F1 females at 10000 ppm (F0 females 14/28, F1 females 3/23).

A statistically analysis using Cohran-Armitage trend test was conducted by RMS to make a decision on adversity of changes in salivary gland. This trend analysis showed a statistically significant trend for the effect of salivary gland with a NOAEL at the lowest dose level of 1000 ppm (see table below):
Trend analysis (Cohran-Armitage Trend Test, exact permutation; StatXact-6):

Males		
F0-Parotid	p-value one sided: 0.0002	NOAEL: 3000 ppm
	p-value two-sided: 0.0004	LOAEL: 10000 ppm
F1-Parotid	p-value one sided: 6.7E-6	NOAEL: 3000 ppm
	p-value two-sided: 1.3 E-5	LOAEL: 10000 ppm
Females		
F0-Parotid	p-value one sided: 0.014	NOAEL: 1000 ppm
	p-value two-sided: 0.027	LOAEL: 3000 ppm
F1-Parotid	p-value one sided: 0.011	NOAEL: 1000 ppm
	p-value two-sided: 0.022	LOAEL: 3000 ppm
F0-submaxillary	p-value one-sided: 0.022	NOAEL: 1000 ppm
	p-value two-sided: 0.045	LOAEL: 3000 ppm
F1-submaxillary	p-value one sided: 0.013	NOAEL: 3000 ppm
	p-value two-sided: 0.026	LOAEL: 10000 ppm

The NOAEL for parental toxicity is proposed to be set at 1000 ppm (66 mg/kg bw/day) based on increased incidence of changes in salivary glands observed at ≥3000 ppm.

NOAEL for offspring and reproductive toxicity was set at 10000 ppm (highest dose level).

The study is acceptable. The study is conducted in accordance with GLP and follows OECD TG 416 (2001) with exception of following deviations, these deviations do not invalidate the study:
(i) relative humidity in experimental animal room was 46%±24% (the guideline recommends the relative humidity to be at least 30%)
(ii) pre-mating oestrous cycles not determined
(iii) pre- and post-implantation loss were not reported
(iv) sperm analysis not performed
(v) uterus, spleen and thyroid of parental animals not weighed
(vi) brain, spleen and thymus of pups not weighed

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

<u>EATS-mediated parameters not investigated:</u>

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- anogenital distance

- oestrous cyclicity
- sperm morphology
- sperm motility
- sperm numbers

Sensitive to but not diagnostic of EATS:

- number of corpora lutea
- pre- and post-implantation loss
- pituitary weight

B.6.6.1/09

Data point	CA 5.6.1/009
Report author	[redacted] *et al.*
Report year	1991
Report title	Dietary range finding study of glyphosate in pregnant rats and their juvenile offspring
Report No.	[redacted] 42/90619
Document No.	Not reported
Guidelines followed in study	Not applicable for this dose-range finding study
Deviations from current test guideline	Missing endpoints: • estrous cycle monitoring • pre-coital interval • sperm analysis • organ weights • monitoring of physical and sexual offspring development. Deviations from the current version of OECD 416 (2001) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 416. Comments by the RMS: Following deviations were observed in addition: • low number of animals used • time-mated F0 females (no mating procedure) • short duration of study • limited histopathology • statistical analyses not performed
Previous evaluation	Yes, evaluated and accepted in DAR (1998), re-evaluated and considered supplementary in RAR (rev 2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Supplementary (See RMS' assessment below)

MATERIALS AND METHODS

Test material:	Glyphosate technical
Lot/Batch:	206-Jak-25-1 Stability of test compound: Stable for at least 5 years
Purity/Radiochemical purity:	Purity: 98.6 % (w/w)
Vehicle:	Plain diet
Animals:	Species: Rat Strain: Sprague-Dawley Crl:CD (SD) BR VAF/Plus Age: Approximately 8 - 10 weeks Sex: Females

Weight at dosing: 214 - 216 g
Acclimation period: 3 days
Diet/Food: Labsure LAD No. 2, *ad libitum*
Water: Tap water, *ad libitum*
Housing: Prior to parturition in groups of five in suspended galvanised metal cages (Bowman ®) equipped with solid sides and back, wire mesh front, floor and top. On Day 20 of pregnancy the rats were re-housed in individual plastic breeding cages (North Kent Plastics, RM-2 type) for the birth and rearing of young. Suitable nesting material was provided. Following weaning, the selected pups were housed by sex, five to a cage. During the first week post weaning plastic cages were used; subsequently, the animals were transferred to metal (Bowman ®) cages.
Environmental conditions:
Temperature: 20-23.5 °C
Humidity: 39-62 %
Air changes: not reported
Light regime: 12/12 hours

STUDY DESIGN

Animal assignment and treatment
In a dose-range-finding study for a subsequent two-generation reproduction study groups of 10 time-mated Sprague-Dawley rats received daily dietary doses of 0, 3000, 10000 and 30000 ppm glyphosate from Day 3 of gestation through gestation and lactation to termination at the end of lactation. These concentrations were equivalent to glyphosate intakes during gestation ranging from 236-311, 799-1010 and 2515-2789 mg/kg bw/day in the low, mid and high dose, respectively. Control animals received the base diet alone. All females were allowed to litter and rear their young to weaning, when 10 male and 10 female offspring per group were selected and reared on their respective diets to six weeks of age.

Diet preparation and analyses
The test material as supplied was weighed out and added to a weighed amount of sieved Labsure Laboratory Diet No.2 and stirred by hand to give a premix of suitable strength. The dietary concentrations required were obtained from this premix by direct dilution with further quantities of diet, homogeneity being achieved by mixing in a rotary double cone blender. Fresh diet was prepared weekly and fed for no more than 7 days from date of preparation and was stored until use in labelled opaque bags.

Diets were not analysed for homogeneity, stability or achieved concentration of the test compound since satisfactory data concerning homogeneity and stability in rodent diet were already available to the Sponsor.

Clinical observations
All animals were regularly handled and observed daily for obvious changes or signs of reaction to treatment.

Body weight
F0 rats:
All adult rats were weighed on Days 1, 3, 6, 10, 14, 17 and 20 of gestation and on Days 0, 7, 14 and 21 *post partum*.

F1 adults:
All animals were weighed on nominal Weeks 4, 5, and 6.

Food consumption and test compound intake
For F0 females, food consumption was measured on a cage basis from 'weigh day' to 'weigh day' up to re-housing on Day 20 of pregnancy.

After weaning, food consumption of selected offspring was measured from nominal Week 4 to termination.

Water consumption
Water consumption was measured daily on a cage basis from allocation to treatment groups to Day 20 of pregnancy.

Reproduction parameters
Pregnancy rate
Pregnancy rate was determined as the percentage of surviving females that were pregnant.

Gestation period
The duration of gestation was recorded. Parturition was observed wherever possible.

Litter data
As soon as possible after parturition the young were counted, individually identified, sexed, weighed and examined for external abnormalities. Pups were weighed on Days 4, 8, 12 and 21 *post-partum* keeping nest disturbance to a minimum, all litters were examined daily for dead and/or abnormal young. After weaning, body weights of selected offspring were recorded on nominal weeks 4, 5 and 6. Dead young were subjected to an autopsy and sexed.

Physical development
Using body weight at Day 21 *post partum* as a basis for selection, ten male and ten female pups per group from an appropriate spread of litters were retained for a further three weeks on treated diet.

Sacrifice and pathology
Approximately one week after birth of litters, apparently non-pregnant females were sacrificed, subjected to *post mortem* examination and their uteri immersed in a 10% solution of ammonium sulphide to reveal evidence of implantation.

On, or shortly after Day 21 post partum, parents and excess pups were sacrificed and examined externally and internally for abnormalities. Sex of the pups was confirmed by gonadal inspection. The uterus of each female which gave birth was visually inspected for implantation sites and the number of sites was recorded. The parotid salivary gland of the parent females was preserved and examined histologically for any microscopic changes.
On, or shortly after Week 6, all surviving Fl animals were sacrificed and subjected to macroscopic *post mortem* examination.

Statistics
Group mean values, except for food and water, were calculated using only the values of animals rearing young to weaning. As F0 animals were group-housed to Day 20, food and water intake were based on all live animals regardless of their pregnancy status.

All derived values (e.g. means, percentage, ratios) were calculated within each litter and the group values derived as a mean of the individual litter values. Statistical analyses were not performed in view of the small group size.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS AND ACHIEVED TEST ITEM INTAKE
Group Analysis of dose formulations was not performed in this dose-range finding study.

F0 females:
Group mean achieved intakes of glyphosate during gestation in groups 2 to 4 ranged from 236-311, 799-1010 and 2515-2789 mg/kg bw/day, respectively. As expected, as the weight of the animals increased the actual intake of glyphosate (expressed as mg/kg bw/day) generally declined. The 3-fold interval in fixed ppm, between the groups remains established when expressed as mg/kg bw/day.

F1 offspring:
Group achieved intakes of glyphosate in Groups 2 to 4, recorded during Weeks 5 and 6, ranged from 368-390, 1291-1335 and 3918-4453 mg/kg/day for males and 355-402, 1191-1271 and 3961-4397 mg/kg/day for females, respectively.

MORTALITY
F0 females:
There were two mortalities. One female at 3000 ppm was sacrificed on Day 22 *post partum* due to poor condition. *Post mortem* examination did not reveal any reason for the apparent dystocia. Since no similar mortalities were seen at higher levels, this death is considered not to be attributable to treatment.

A second animal at 30000 ppm was found dead on Day 21 *post partum* (Day 43 of study). Post mortem examination or signs prior to sacrifice did not reveal the cause of death. It cannot be finally concluded whether this mortality was due to treatment with the test item or not.

F1 offspring:
No mortalities were noted from weaning until scheduled sacrifice at 6 weeks of age.

CLINICAL OBSERVATIONS
F0 females:
Clinical signs associated with the test item included soft faeces and yellow stained sawdust (considered to be caused by the urine) in both cages of animals at 10000 and 30000 ppm. Onset of these signs was earlier at 30000 ppm than at 10000 ppm (soft faeces occurred immediately after dietary administration commenced at 30000 ppm but not until the third week *post partum* at 10000 ppm, yellow staining of the sawdust occurred on Day 26 *post coitum* at 30000 ppm but not until Day 28 *post coitum* at 10000 ppm), with signs still apparent in both groups at termination. There were no clinical signs at 3000 ppm considered to be attributable to the test item.

Table B.6.6.1/06-01: F0 adult performance and clinical signs

Clinical sign	Control (0 ppm)	Low (3000 ppm)	Mid (10000 ppm)	High (30000 ppm)
Performance (no. of females)				
Mated	10	10	10	10
Non-pregnant	1	0	0	1
Sacrificed/deceased	0	1	0	1+
Rearing young to weaning	9	9	10	9+
Clinical signs (no. of females affected)				
Soft faeces	-	-	10	10
Yellow stained sawdust	-	-	10	10
Pale extremities	-	1++	-	-
Hair loss/scabbing on face	2	-	-	-

+ animal died Day 21 *post partum*, litter reared to weaning
++ animal subsequently sacrificed

F1 offspring:
There were no mortalities. Soft faeces were observed for all animals at 30000 ppm from Week 4 through to sacrifice at Week 6. No other clinical signs were observed at this or lower dosages.

BODY WEIGHT
F0 females:
At 30000 ppm, the rate of body weight gain following the initiation of treatment was reduced to Day 14 of pregnancy; thereafter, the rate of body weight gain to Day 20 of pregnancy was similar to controls, however, absolute parity with controls at Day 20 was not achieved. At both 3000 and 10000 ppm the pattern of body weight gain during pregnancy was essentially similar to controls throughout, although by Day 14 of pregnancy, body weights were slightly lower than controls.

During the first week of lactation the pattern of body weight change in all groups was similar. Thereafter, slight differences in the pattern of change were apparent to weaning at 10000 and 30000 ppm in so much as more weight loss occurred than in the control group. There were no further effects at 3000 ppm.

Table B.6.6.1/06-02: F0 females - body weights and body weight changes during gestation (Group mean values)

Dietary concentration (ppm)	No. of animals	Gestation Day						
		1	3	6	10	14	17	20
	Body weights (g) at Day							
0 (Control)	9	187.7	215.2	241.6	280.1	318.4	352.4	406.9
3000	9	186.8	216.4	246.0	277.2	311.2 (2%)	343.6	393.9
10000	10	185.4	214.5	241.1	277.8	309.9 (3%)	346.4	404.6
30000	9	187.0	215.8	236.0 (2%)	266.0 (5%)	296.7 (7%)	330.0 (6%)	385.6 (5%)
		Bodyweight change (g) relative to gestation Day 3						
0 (Control)	9	-27.6	0.0	26.3	64.9	103.2	137.2	191.7
3000	9	-29.7	0.0	29.6	60.8	94.8 (8%)	127.1	177.4
10000	10	-29.1	0.0	26.6	63.3	95.4 (8%)	131.9	190.1
30000	9	-28.8	0.0.	20.2 (23%)	50.2 (23%)	80.9 (22%)	114.2 (17%)	169.8 (11%)

Table B.6.6.1/06-03: F0 females - body weights and body weight changes during lactation (Group mean values)

Dietary concentration (ppm)	No. of animals	Lactation Day			
		0	7	14	21
	Body weights (g)				
0 (Control)	9	315.4	339.1	344.6	335.0
3000	9	299.3	329.2	326.7	311.1
10000	10	309.8	338.0	330.1	322.5
30000	8+	292.5 (7%)	321.0 (5%)	300.0 (13%)	286.9 (14%)
		Body weight change (g) relative to gestation Day 3			
0 (Control)	9	100.2	123.9	129.3	119.8
3000	9	82.9	112.8	110.2	94.7
10000	10	95.3	123.5	115.6	108.0
30000	8+	72.4 (28%)	100.4 (19%)	79.1 (39%)	62.9 (47%)

+ excludes one animal found dead Day 21

F1 offspring:
For pup body weight development please refer to “Litter data”.

At 30000 ppm the body weight was reduced in male and female offspring retained to 6 weeks of age

Table B.6.6.1/06-04: F1 generation - body weights Week 4 –to 6 (Group mean values)

Dietary concentration (ppm)		No. of animals	Nominal age (days)			
			21	28	35	42
		Body weights (g)				
		Males				
(Control)		10	50	95	156	226
3000		10	47 (6%)	90 (5%)	151 (3%)	218 (4%)
10000		10	44 (12%)	86 (9%)	143 (8%)	210 (7%)
30000		10	31 (38%)	66 (31%)	115 (26%)	169 (25%)
Females						
0 (Control)		10	48	89	137	175
3000		10	44 (8%)	83 (7%)	130 (5%)	166 (5%)
10000		10	42 (12%)	80 (10%)	129 (6%)	167 (5%)
30000		10	31 (35%)	64 (28%)	109 (20%)	148 (15%)

FOOD CONSUMPTION

F0 females:

Apart from a slight reduction in food intake during the first three days of treatment at 30000 ppm, food intake of all treated groups was similar to concurrent controls throughout pregnancy.

Table B.6.6.1/06-05: F0 females- food consumption during gestation (Group mean values)

Dietary concentration (ppm)	No. of animals	Gestation Days				
		3-5	6-9	10-13	14-16	17-19
	Food consumption (g/rat/day)					
0 (Control)	10	23	26	27	28	30
3000	10	24	26	27	28	29
10000	10	23	26	26	28	30
30000	10	21	25	27	28	30

Offspring retained to six weeks of age (F1 generation):

Food consumption at 30000 ppm was lower than controls during Weeks 5 and 6 (males only). There were no other effects considered attributable to treatment for males or females at any dosage. In addition, the food conversion ratio for males at 30000 ppm was slightly greater when compared to controls (Week 6 only), indicating a slightly lower efficiency of food utilisation into body weight gain. There were no other effects on food conversion ratios.

Table B.6.6.1/06-06: F1 offsprings - food consumption in Weeks 5 and 6 (Group mean values)

Dietary concentration (ppm)	No. of animals	Week	
		5	6
	Food consumption (g/rat/week)		
	Males		
0 (Control)	10	106	168
3000	10	104	168
10000	10	104	165
30000	10	83 (22%)	148 (12%)

Dietary concentration (ppm)	No. of animals	Week	
		5	6
	Females		
0 (Control)	10	91	132
3000	10	100	123
10000	10	87	132
30000	10	80	132

WATER CONSUMPTION

At 30000 ppm, slight increases in water intake during gestation were apparent towards the end of pregnancy when compared with concurrent control values. At 3000 and 10000 ppm, no adverse effects on water consumption were observed.

Table B.6.6.1/06-07: Water consumption during gestation (Group mean values)

Dietary concentration (ppm)	No. of animals	Gestation Days				
		3-5	6-9	10-13	14-16	17-19
	Water consumption (g/rat/day)					
0 (Control)	10	32	35	37	41	44
3000	10	32	33	36	41	45
10000	10	28	31	34	37	42
30000	10	30	34	38	43	49 (11%)

REPRODUCTIVE PARAMETERS

Pregnancy Rate and Duration of Gestation

The pregnancy rate was high, 90, 100 (incl. one, dam that died on day 21 *post partum*), 100 and 90% in the control and the low, mid and high dose group, respectively. The duration of pregnancy was similar in all groups and not adversely affected by treatment with glyphosate.

Mating Performance, Fertility and Gestation

There were no treatment-related effects on mating performance, fertility and gestation length for both F0 and F1 generation animals.

Table B.6.6.1/06-08: performance and gestation parameters

	Control (0 ppm)	Low (3000 ppm)	Mid (10000 ppm)	High (30000 ppm)
Performance (no. of females)				
Mated	10	10	10	10
Non-pregnant	1	-	-	1
Sacrificed/deceased	0	1+	0	1++
Pregnant	9	9	10	9
Rearing young to weaning	9	9	10	9+
Mean duration of gestation (days)	21.3	21.6	21.4	21.3
No. of implantation sites	13.8	15.0	15.0	15.3
Pre-birth loss (%)	3.6	5.3	5.8	5.0

+ sacrificed on Day 22 of pregnancy due to poor condition
++ animal died Day 21 post partum, litter reared to weaning

LITTER DATA

Size and Viability

A total of 9, 9, 10 and 9 females reared litters to weaning in Groups 1 to 4, respectively (including one female found dead at weaning in Group 4).

The implantation rates in all treated groups were higher than the controls. Since pup losses, both pre-birth and from birth to weaning, were generally similar among the groups, litter size of all treated groups was, as a consequence, generally greater than controls throughout weaning. These findings are not, however, considered to be an adverse effect of treatment. Glyphosate did not selectively affect pups of one sex since, in all groups, sex ratios at birth and weaning were similar. The mean sex ratio did not reveal any treatment-related effect. In the mid dose group, a slightly higher percentage of male pups was noted both at birth and on day 21 *post partum* (60.5% compared to 46.7% in the control). In the absence of a dose-relationship this finding was considered incidental.

Growth and Development
At 30000 ppm, mean pup weight was reduced at birth and diverged further away from controls through to Day 21 when mean pup weight at this level was only 62% of the controls.

At 3000 and 10000 ppm, mean pup weight was initially (at birth and on day 4 post partum) similar to the controls but diverged below control values to an extent that on Day 21 *post partum* mean pup weight at 3000 and 10000 ppm was 9% and 13% lower than the corresponding control value.

Table B.6.6.1/06-09: Litter data of animals rearing young to weaning- group mean values- F0 generation

	Control (0 ppm)	**Low (3000 ppm)**	**Mid (10000 ppm)**	**High (30000 ppm)**
Mean litter size				
at birth	13.2	14.2	14.1	14.6
at day 4 *post partum*	12.2	13.8	14.0	14.1
at day 8 *post partum*	12.1	13.7	13.6	13.8
at day 21 *post partum*	12.1	13.6	13.5	13.2
Mean pup weight (g)				
at birth	6.6	6.6	6.6	6.3 (4.5%)
at day 4 *post partum*	10.1	9.9	9.9	9.3 (8%)
at day 8 *post partum*	17.6	16.3	16.6	14.8 (16%)
at day 21 *post partum*	49.3	45.1 (9%)	43.1 (13%)	30.8 (38%)
Percentage male pups (%)				
at birth	46.7	50.0	60.5	47.4
At day 21 *post partum*	48.0	50.2	61.5	47.5

PATHOLOGY
Necropsy F0 females
Watery and/or dark contents in the gastro-intestinal tract were observed in 0, 2, 7 and 8 animals in Groups 1 to 4 respectively. Distended and/or congested stomach was seen in 0, 2, 5 and 4 animals and distended caecum in 0, 0, 0 and 4 animals in Groups 1 to 4 respectively. These findings generally followed the trend noted in the clinical signs observed.

There were no other macroscopic changes considered to be related to treatment.

Table B.6.6.1/06-10: F0 females - necropsy findings

Clinical sign	**Control (0 ppm)**	**Low (3000 ppm)**	**Mid (10000 ppm)**	**High (30000 ppm)**
Necropsy findings				
Fur, ventral surface: yellow stained	-	-	1	-
Gastro-intestinal tract: contents watery and/or dark	-	2	7	8
Stomach: distended and/or congested	-	2	5	4
Stomach: contents watery	1	2	-	1
Caecum: distended	-	-	-	4
Caecum: contents watery	1	-	-	1
Salivary glands: enlarged /firm/congested/swollen	-	2	6	8
Lungs: multiple pale subpleural foci	1	3	3	1

Clinical sign	Control (0 ppm)	Low (3000 ppm)	Mid (10000 ppm)	High (30000 ppm)
Uterus: severe fluid distension	1*	-	-	-

* animal not pregnant

Post mortem examination of weanlings (F1 generation)
Four pups with congested salivary glands were observed at 10000 ppm and one pup with congested salivary glands was observed at 3000 ppm. Since no similar findings were seen at 30000 ppm the significance of these incidences is unclear.

Neither the incidence nor the type of other macroscopic findings were considered to be attributable to treatment according to study author.

Histopathology
F0 females:
A dose-related incidence and degree of granular basophilic cytoplasm of acinar cells of the parotid glands was seen with 0, 2, 0 and 0 animals showing minimal effects, 0, 0, 2 and 0 animals showing moderate effects and 0, 0, 8 and 9 animals showing marked effects in Groups 1 to 4 respectively. This change was associated with hypertrophy of acinar cells with 0, 2, 2 and 0 animals with minimal hypertrophy and 0, 0, 8 and 9 animals in Groups 1 to 4 respectively with moderate hypertrophy of the acinar cells. Prominent mitoses were also seen in 2 animals at 30000 ppm, but not at lower treatment levels or the controls.

Table B.6.6.1/06-11: F0 females - microscopic findings

Microscopic findings	Control (0 ppm)	Low (3000 ppm)	Mid (10000 ppm)	High (30000 ppm)
No. of animals examined	9	9	10	9
Parotid salivary glands				
Granular basophilic cytoplasm of acinar cells:				
Minimal	-	2	-	-
Moderate	-	-	2	-
Marked	-	-	8	9
Hypertrophy of acinar cells:				
Minimal	-	2	2	-
Moderate	-	-	8	9
Prominent mitosis in acinar cells	-	-	-	2

Offspring retained to six weeks of age (F1 generation):
Post mortem examination revealed swollen/enlarged parotid salivary glands in 5/10 males and 2/10 females at 30000 ppm, and in 1/10 males at 3000 ppm. Soft gastro-intestinal contents were noted in 7/10 males and 9/10 females at 30000 ppm. Grey/blue contents of the jejunum were noted in 2/10 females at this dosage.

CONCLUSION BY STUDY AUTHOR
Assuming that effects seen in this preliminary study are representative of those that would occur in a comparable multiple generation study, a possible high level for the main study would appear to be less than 30000 ppm but greater than 10000 ppm, with a low level being less than 3000 ppm.

Assessment and conclusion by applicant:
Dietary administration of glyphosate to pregnant dams at 30000 ppm resulted in a single death, clinical signs and body weight effects; at 10000 ppm resulted in clinical signs and body weight effects and at 3000 ppm slightly lower body weight gain by day 14 of pregnancy. Findings in F1 pups at 30000 ppm resulted in clinical signs, reduced food intake and body weight effects. While dose dependent changes were observed in the parotid glands of the parents and offspring these findings are considered to be an adaptive response due to oral irritation from the ingestion of glyphosate, an organic acid, in the diet and as such not considered to be toxicologically relevant. The doses selected for the definitive study were 0, 1000, 3000 and 10000 ppm.

Assessment and conclusion by RMS:

Groups of 10 time-mated Sprague-Dawley rats received daily dietary doses of 0, 3000, 10000 and 30000 ppm glyphosate from Day 3 of gestation through gestation and lactation to termination at the end of lactation. All females were allowed to litter and rear their young to weaning, when 10 male and 10 female offspring per group were selected and reared on their respective diets to six weeks of age. No adverse effects on reproduction parameters nor on survival of pups through weaning were observed in this study. However, it could be noted that the study was limited (few animals, limited parameters investigated).

Treatment was associated with following findings:

- Mortality observed at 30000 ppm (one F0 animal died on Day 21 *post partum*, cause of death not identified)
- Clinical signs: Soft faeces and yellow stained sawdust were observed in adult F0 females at ≥10000 ppm. Soft faeces was observed in offspring retained to six weeks of age at 30000 ppm
- Increased water consumption towards the end of pregnancy observed at 30000 ppm (F0 females: 11%)
- Reduced body weight gain during pregnancy/lactation observed F0 females at 30000 ppm (Gestation: up to 23%; Lactation: up to 47%). Slightly reduced body weight gain by Day 14 of pregnancy observed in F0 females at 3000 and 10000 ppm (reduction of 2-3% compared to control)
- Reduced weight gain in offspring retained to six weeks of age (F1 generation) at 30000 ppm (Day 42: males: 25%, females: 15%)
- Reduced food consumption in offspring retained to six weeks of age (F1 generation) during Weeks 5 (22%) and 6 (12%) (30000 ppm group males only)
- Reduced mean pup weights (Day 21 *post partum*: 30000 ppm: 38%; 10000 ppm: 13%, 3000 ppm: 9%)
- Macroscopic salivary gland changes (enlarged/firm/congested/swollen) observed in F0 females (at 30000 ppm: 8/9, at 10000 ppm: 6/10, at 3000 ppm: 2/9)
- Macroscopic changes in parotid salivary gland (enlarged/swollen) observed in offspring retained to six weeks of age at 30000 ppm (males (5/10), females (2/10) and at 3000 ppm (1/10 male)
- Macroscopic changes in salivary gland (congested) observed in one pup at 3000 ppm and in four pups at 10000 ppm (significance unclear)
- Macroscopic gastro-intestinal changes observed in F0 females at ≥3000 ppm (At 3000 ppm: content watery and/or dark (2/9), stomach distended and/or congested: (2/9); At 10000 ppm: content watery and/or dark: (7/10), stomach distended and/or congested: (5/10); At 30000 ppm: content watery and/or dark: (8/9), stomach distended and/or congested: (4/9), distended caecum (4/9))
- Macroscopic gastro-intestinal changes (soft content) observed in offspring retained to six weeks of age at 30000 ppm (males: 7/1), females: 9/10)
- Microscopical changes in salivary gland in F0 females at ≥3000 ppm (At 30000: marked granular basophilic cytoplasm of acinar cells and moderate hypertrophy of acinar cells (9/9), prominent mitoses in acinar cells (2/9); At 10000 ppm: moderate/marked granular basophilic cytoplasm of acinar cells and minimal/moderate hypertrophy of acinar cells (10/10); At 3000 ppm: minimal granular basophilic cytoplasm of acinar cells with minimal hypertrophy of acinar cells (2/9).

The study is acceptable as supplementary data only. The study is not suitable for NOAEL setting (low number of animals used, limited parameters investigated, no statistics). However, it is noteworthy that effects occurred in this study at lower dose levels than in the main study. The findings of reduced pup weights observed at the low dose level of 3000 ppm were not confirmed in the main study using sufficient amount of animals and test doses up to 10000 ppm, nor in other available generational studies in which much more animals were employed.

The study was checked for compliance with OECD TG 416 (2001) and following deviations were observed:
(i) only 10 females/group were used (the guideline recommends 20 pregnant females/group)
(ii) the F0 females were time-mated (the guideline recommends a mating procedure)
(iii) duration of study was only 10 weeks. F0 exposed from Day 3 of pregnancy through the termination of the study, females were allowed to litter and rear their young to weaning, when 10 males and 10 female offspring per group were selected and reared on their respective diets to six weeks of age (the guideline recommends that F0 animals are dosed at least 10 weeks before mating period, dosing continued in both sexes during the 2 week mating period and continued throughout pregnancy and up to the weaning of the F1 offspring. The same procedure for the F1 offspring to produce the F2 generation)
(iv) oestrous cycle not evaluated

(v) litter parameters limited
(vi) sperm parameters not evaluated
(vii) sexual maturation not investigated
(viii) no organ weighed
(ix) histopathology limited (salivary glands investigated only)
(x) statistical analyses not performed

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- sperm morphology
- sperm motility
- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- epididymis histopathology
- epididymis weight
- liver weight
- ovary histopathology
- ovary weight
- prostate histopathology
- prostate weight
- seminal vesicles histopathology
- seminal vesicles weight
- testis histopathology
- testis weight
- uterus histopathology
- uterus weight
- vagina histopathology
- vaginal smears

Sensitive to but not diagnostic of EATS:

- number of corpora lutea
- time to mating
- adrenal weight
- pituitary weight
- pre-and postimplantation loss

B.6.6.1/10

Data point	CA 5.6.1/010
Report author	[redacted].
Report year	1990
Report title	Two Generation Reproduction Feeding Study with Glyphosate in Sprague-Dawley Rats

Report No.	[redacted]-10387
Document No.	Not reported
Guidelines followed in study	Not stated, but in general accordance with OECD 416 (1983)
Deviations from current test guideline	• no data on food efficiency • no details on fertility indices, number of live births and post-implantation loss, number of pups with grossly visible abnormalities • no sperm analysis • no determination of oestrus cycle length • no determination of physical or sexual development landmarks • most parental animal organ weights missing • several offspring organ weights missing Comments by the RMS: Following deviations were observed in addition: • minor deviations in housing conditions: Temperature was 18-26ºC (the guideline recommends that the temperature in the experimental animal room should be 22 ± 3°C) • coagulating gland and cervix not included in the histopathological examination Only ovaries and testes with epididymides weighed (F0 and F1a adults) (uterus, prostate, seminal vesicles, brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands not weighed). Brain, spleen and thymus of pups not weighed. No details on pre-and postimplantation loss
Previous evaluation	Yes, evaluated and accepted in DAR (1998) and RAR (2015)
GLP/Officially recognised testing facilities	Yes, conducted under GLP
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material: Glyphosate
Lot/Batch: XLI-203
Stability of test compound: Not reported
Purity/Radiochemical purity: Purity: 97.67 %
Vehicle: Plain diet
Animals: Species: Rat
Strain: Albino Rat
Age: Approximately 7 weeks (F0 adults)
Sex: Males and females
Weight at dosing: Males: 165 – 207.6 g; Females: 135.6 – 162.7 g
Acclimation period: No data
Diet/Food: Purina Mills Certified RODENT CHOW No. 5002, *ad libitum*
Water: St. Louis public water, *ad libitum*
Housing: Housing for premating and gestation (day 0 through 13): individual suspended stainless steel cages over paper bedding; during mating females were housed in the male´s cages
Housing for gestation and lactation (from day 14 of gestation through lactation): females housed in double wide cages with solid bottoms and wood shavings for bedding
Environmental conditions:
Temperature: 18-26 °C
Humidity: 40-70%
Air changes: not reported
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In a two-generation reproduction study, groups of 30 Sprague-Dawley rats per sex of the F0 generation received daily dietary doses of 2000, 10000 and 30000 ppm (corresponding to 132-140, 666-711, 1983-2230 mg/kg bw/day for males and 160-163, 777-804, 2322-2536 mg/kg bw/day for females (calculated from F0 and F1a adults)) glyphosate in the diet. After 11 weeks of treatment pairing of animals within each dose group was undertaken on a one male: one female basis in a male´s cage for 7 days, to produce the F1a litters. If there was no evidence of mating after 7 days (copulatory plug, or vaginal smear), the female was co-housed with a male having recorded copulatory activity for additional 7 days, or until copulatory evidence was found. For F0 and F1 generation, gestation day 0 was set on the day on which copulatory evidence was found and lactation day 0 the day on which delivery of pups was completed.

At weaning of offspring from the F0 mating phase, groups of 30 males and 30 females offspring from each dose group were selected to form the F1 generation and the mating procedure for F1a adults was conducted in the same way except modifications to exclude sibling matings. The remaining surviving F0 females and unselected offspring were terminated at Day 21 *post partum*. F0 males were killed at completion of mating phase. The offspring selected for the F1a generation were dosed for approximately 14 weeks and then mated to produce the F2a and F2b litters (a second mating of the F1 generation was performed due to reduced litter sizes in pups from F0 of the 30000 ppm dose group). At weaning of the F2 litters all surviving adults and their offspring were killed, whereas F1 males were sacrificed after completion of mating phase.

Diet preparation and analyses
Approximately each week (except in one week when diets were prepared twice the same week and not during the following week) a known amount of glyphosate was mixed with the diet for 10 minutes in a HOBART HCM-450 mixing machine to achieve a batch size of 18 kilograms at each dose level.

The stability and homogeneity of the test substance in the diet were determined by liquid chromatography of duplicate samples from top, middle and bottom of mixer from the lowest and highest dietary levels stored in an open container at ambient temperature for 6 and 14 days or when frozen in a closed container for 35 days.

Clinical observations
A detailed observation for signs of toxicity was performed once weekly for the adult animals and for the offspring on days of weight measurement.

Body weight
Adult male animals of the F0 and F1a generation were individually weighted once weekly. The same was done for the female animals until copulation was confirmed, then females were weighted on days 0, 7, 14, and 21 of gestation and lactation.

Offspring was weighted on days 0, 4 (pre- and post-culling), 14 and 21 of lactation (except F1a males approximately two weeks prior to sacrifice and F1a females for approximately three weeks prior to mating for the F2b generation).

Food consumption and compound intake
Food consumption was recorded weekly for F0 and F1a adult males, except during mating, and also weekly for adult F0 and F1a female animals until mating. After confirmed copulation, the maternal food consumption was monitored for days 0-7, 7-14 and 14-21 of gestation and lactation, but it was not determined for females approximately three weeks prior to mating for the F2b generation and generally not for female animals that did not became pregnant.

Food conversion efficiency was not calculated.

Water consumption
No data on water consumption was given in the report.

Pregnancy and parturition
Data on total paired females, females with confirmed copulation/total paired, pregnant/total paired, pregnant/ confirmed copulation was monitored as well as precoital (for pregnant animals) and gestational length in days. For males, the following items of interest were given: males with confirmed copulation/total paired, males impregnating females/total paired and males impregnating females/confirmed copulation.

Litter data
The following litter data were recorded: Litter size, dead pups/litter, mean pup weight (on day 0, 4 (pre-/post-cull), 14, 21) and survival (%).

Physical and sexual development
No details on physical and sexual development of the offspring was reported.

Sacrifice and pathology
All adult animals, which died or were sacrificed in moribund condition were subjected to a gross necropsy and selected tissues were sampled. Pups found dead or culled pups also underwent gross pathology, but no tissues were saved. No organ weights were determined.

All F1a weanlings, that were not selected for mating, F2a and F2b weanling pups as well as females which had littered on or after 21 of lactation were sacrificed as scheduled. Non-pregnant adult females were killed at least 5 days after last expected parturition date and adult males after completion of the mating phase.

External and internal cavities of the dead animals were opened and the organs were examined in place and then removed. Hollow organs were opened and examined. The following organs of F0 and F1a males and females from each dose group that were sacrificed at the end of the study sampled, were weighed: ovaries and testes with epididymides. When present, the following organs from the F0 and F1a adults (unscheduled deaths and scheduled sacrifice) were retained: kidneys, ovaries, prostate, seminal vesicle, skin/mammary gland, testes, epididymis, uterus/vagina and gross lesions (pituitary retained for F1a adults only). Tissues from the F1a weanlings were saved at the discretion of the necropsist. From the F2a and F2b weanlings, which where sacrificed at schedule, the kidneys of 1 pup per sex and litter were saved.

A histopathological examination was performed on all sampled tissues from all F0 and F1 control and high-dose animals, and on one F2b weanling/sex/litter (selected at random) as well as on all retained tissues from unscheduled adult deaths. For preparation, fixed tissues were washed, dehydrated, embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined under light microscopy.

Statistics
Dunnett´s multiple comparison test (two-tailed) was used to detect statistically significant differences in adult body weights and food consumption between treated animals and their respective control.

Terminal body weights, maternal body weights and food consumption during gestation and lactation, pup weights, precoital length, gestational length, litter size, dead pups/litter, pup survival, absolute organ weights and organ/body weight ratios were evaluated by decision-tree statistical analyses procedures which, depending on the results of tests for normality and homogeneity of variance [Bartlett's Test], were chosed either parametric [Dunnett's Test and Linear Regression] or nonparametric [Kruskal-Wallis, Donckheere's and/or Mann-Whitney Tests] routines to detect differences and analysed for trend.

The uncorrected Chi-Square test was used to examine fertility indices, e.g. females/males with confirmed copulation/total paired, pregnant/confirmed copulation (females) and males impregnating females/total paired as well as males impregnating females/confirmed copulation.

Fisher´s Exact test with Bonferroni Inequality Procedure was used for statistical analysis of microscopic lesions.

Other statistical routines used for some data included: Bartlett's Test to evaluate homogeneity of variances, Analysis of Variance to determine if the sample (group) means could be considered as an estimate of a common population, and Grubb's Test to detect outliers.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS

The analysis of the test substance stability conducted over the time span of the study indicated that the test material was stable in the diet and homogeneity was adequate for study use. The stability of the test material in the diet was demonstrated at the low and high dose level, stored in an open container at ambient temperature for 6 and 14 days, or when frozen in a closed container for 35 days.

Analysis for achieved concentrations, demonstrated that the test substance-levels in the prepared diet were in the range of 95 to 96.7% of the nominal concentration.

TEST COMPOUND INTAKE

The group mean achieved dosages are summarised in the table below.

Table B.6.6.1/07-01: Group mean compound intake levels during pre-mating periods of F0 and F1

Dose group	Dietary concentration (ppm)	Mean daily test substance intake (mg/kg bw/day)*			
		F0		F1	
		Males	Females	Males	Females
control	0	0	0	0	0
low	2000	132	160	140	163
mid	10000	666	777	711	804
high	30000	1983	2322	2320	2536

* based on actual food intake and body weight data; values were calculated in the report

MORTALITY

There were no treatment-related mortalities.

One female of the F0 generation at 10000 ppm died early in the study. This animal was never mated and at necropsy changes in bladder in kidneys were observed. One male animal in each of the 2000 and 30000 ppm dose groups (F1 generation) died. Necropsy of these animals noted thymus and respiratory changes. One female animal of the F1 generation (2000 ppm) was sacrificed in extremis and another female (same generation, same dose group) died. Kidney changes and retained foetus; pups in uterus and stomach changes, respectively, were observed in these two females.

Concerning the offspring, dead pup counts at day 0 and survival of all F1a, F2a and F2b treated pups were not adversely affected when compared to the controls.

CLINICAL OBSERVATIONS

The only clinical signs that were related to the test substance were soft stool in the animals of the 30000 ppm dose group. Other clinical signs, such as red ocular discharge/laboured respiration/overgrown teeth/piloerection/abrasions/emaciated and dehydrated appearance/misuse of limbs/focal loss of hair/swollen feet, occurred sporadically and were not considered to be treatment related.

BODY WEIGHT

At the highest exposure level of 30000 ppm, reduced body weights were observed in both sexes and in the F0 and F1 generation. In the F0 generation, body weight gains gradually decreased over time to approximately 8% less than controls prior to mating. F0/F1 weaning animals were lighter in weight than their corresponding controls and maintained that weight difference (approx. 10% less than control) until the end of the study.

No test-substance related body weight effects were observed in the adult animals of the 2000 and 10000 ppm dose groups prior to mating.

During gestation and lactation, maternal body weights in the highest dose group tended to remain lower than in controls, but the animals showed a rather greater body weight gain than the controls during gestation and lactation so that by the end of lactation, body weights were approximately the same as those of the controls.

Terminal body weights were significantly decreased for both sexes at the highest exposure level.

Table B.6.6.1/07-02: Selected mean group body weights

Dietary concentration (ppm)	No. of animals		Mean group body weight (g) at Day					
			0	72	T#	0	72	T#
			F0 Generation					
			Males			Females		
0 (Control)	30	mean	187.9	494.6	549.56	150.5	276.7	296.31
		SD	11.65	34.86	46.76	6.86	23.85	23.63
2000	30	mean	188.1	497.6	550.19	150.5	272.6	290.64
		SD	11.35	49.87	80.72	7.03	22.86	19.50
10000	30	mean	188.1	484.4	539	150.2	273	290.71
		SD	11.57	42.13	58.13	7.04	27.92	25.35
30000	30	mean	188	455.8**	503.51**	150.3	253.8**	265.91
		SD	11.56	46.46 (8%)	45.66 (8%)	7.06	18.46 (8%)	15.44
			F1 Generation					
			129	219	T#	128	219	T#
0 (Control)	30	mean	118.3	534.7	625.04	99.8	285.8	316.21
		SD	26.11	38.84	53.11	17.44	27.63	37.37
2000	30	mean	115.2	540.3	632.14	96.7	282.1	313.74
		SD	16.2	44.9	74.57	11.47	24.5	30.53
10000	30	mean	114.8	514.1	590.98	97.1	275.9	312.36
		SD	17.42	58.31	70.06	14.18	20.55	26.71
30000	30	mean	104.9*	483.4**	543.40**	88.8*	253.7**	284.72**
		SD	19.79 (11%)	41.32 (10%)	58.12 (13%)	16.32 (11%)	19.56 (11%)	18.04 (10%)

*: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.05)
**: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.01)
#T: Termination

Table B.6.6.1/07-03: Mean maternal body weights during gestation

Dietary concentration (ppm)	No. of animals		Mean group body weight (g) at Day (Gestation)			
			0	7	14	21
			F0 Generation			
0 (Control)	24	mean	274	301.83	324.41	398.26
		SD	24.26	24.58	22.85	26.12
2000	29	mean	272.72	297.33	319.90	392.86
		SD	20.52	21.71	19.84	24.28
10000	28	mean	271.80	299.22	323.43	395.08
		SD	24.12	26.40	28.44	25.87
30000	28	mean	255.05**	282.44**	305.83**	375**
		SD	16.49 (7%)	15.27 (6%)	17.44 (6%)	24.70 (6%)
			F1 Generation (First Mating)			
			0	7	14	21
0 (Control)	24	mean	285.29	308.95	328.70	392.56
		SD	25.48	26.58	29.18	36.19
2000	29	mean	278.65	304.40	324.15	383.45
		SD	23.42	23.48	25.06	28.18
10000	28	mean	268.89*	297.23	319.08	382.71
		SD	19.24 (6%)	18.81	19.27	21.77
30000	28	mean	251.30**	278.28**	299.48**	360.46**
		SD	17.42 (12%)	18.92 (10%)	19.29 (9%)	33.31 (8%)
			F1 Generation (Second Mating)			

Dietary concentration (ppm)	No. of animals		Mean group body weight (g) at Day (Gestation)			
			0	7	14	21
			0	7	14	21
0 (Control)	24	mean	324.22	340.99	363.44	428.99
		SD	23.11	27.81	27.98	36.87
2000	29	mean	315.21	338.27	360.35	426.88
		SD	26.06	28.67	28.39	33.67
10000	28	mean	305.27*	333.66	357.50	428.51
		SD	20.26 (6%)	22.45	24.49	26.17
30000	28	mean	281.46**	308.92**	330.95**	393.67**
		SD	17.79 (13%)	22.19 (9%)	22.36 (9%)	34.88 (8%)

*: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.05)
**: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.01)

Table B.6.6.1/07-04: Mean maternal body weights during lactation

Dietary concentration (ppm)	No. of animals		Mean group body weight (g) at Day (Lactation)			
			0	7	14	21
			F0 Generation			
0 (Control)	24	mean	299.96	319.59	317.33	313.39
		SD	23.21	23.58	28.96	20.01
2000	29	mean	297.48	317.91	314.53	313.96
		SD	21.10	18.66	25.22	16.63
10000	28	mean	298.78	315.15	312.41	319.10
		SD	20.81	22.04	22.94	18.61
30000	28	mean	285.84*	307.64	304.75	316.68
		SD	13.91 (5%)	12.48	20.68	15.43
			F1 Generation (First Mating)			
			0	7	14	21
0 (Control)	24	mean	299.29	313.60	337.68	313.49
		SD	27.02	26.12	25.31	21.38
2000	29	mean	295.16	308.28	332.10	314.69
		SD	23.58	22.56	23.92	23.95
10000	28	mean	296.63	310.80	328.29	313.14
		SD	19.01	18.64	18.33	14.06
30000	28	mean	277.91**	289.88**	315.88**	306.15
		SD	17.89 (7%)	17.23 (8%)	17.47 (6%)	20.18
			F1 Generation (Second Mating)			
			0	7	14	21
0 (Control)	24	mean	342.78	343.21	353.34	337.16
		SD	32.46	27.11	21.15	17.22
2000	29	mean	340.16	336.62	348.40	331.96
		SD	28.54	16.11	25.89	20.67
10000	28	mean	333.80	342.41	352.70	334.56
		SD	23.35	26.93	20.43	13.82
30000	28	mean	312.39**	324.09*	337.08	329.95
		SD	23.73 (9%)	20.50 (6%)	19.09	18.41

*: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.05)
**: Dunnett´s test (two-tailed) indicates statistically significant difference (p<0.01)

FOOD CONSUMPTION
Overall, food intake was not notably affected during the study.

All animals of the 30000 ppm dose group consumed about 1 to 2 grams/day less than controls. This effect was most pronounced in the first week of exposure and also observed in the F0 dams. Subsequent dams (F1 first and second matings) tended to eat similar or larger amounts of the diet than controls.

No effects on food consumption were observed in the animals of the 2000 and 10000 ppm dose groups.

REPRODUCTIVE PARAMETERS

Mating Performance, Fertility, Gestation and Lactation

No effects on mating and fertility rates were observed in the F0 and F1 dams when compared to controls and no effects were observed on precoital length or length of gestation at any treatment level.

Table B.6.6.1/07-05: Mating, fertility and gestation parameters (F0)

	Dietary concentration (ppm)			
	0	**2000**	**10000**	**30000**
# of females	30	30	29	30
Females with confirmed copulation /Total paired	96.7%	100%	100%	100%
Pregnant total/Paired	80%	98.7%	96.6%	93.3%
Mean precoital time, days (% of control)	3.58	3.41 (95%)	3.07 (86%)	3.74 (104%)
# of pregnant animals/total number	24/30	27/30	28/29	27/30
Males with confirmed copulation/total paired	86.7%	93.3%	93.1%	90.0%
Males impregnating females/total paired	70.0%	90.0%	89.7%	83.3%
Gestational length (days) (% of control)	22.3	22.2 (100%)	22.5 (101%)	22.3 (100%)

Table B.6.6.1/07-06: Mating, fertility and gestation parameters (F1A First Mating)

	Dietary concentration (ppm)			
	0	**2000**	**10000**	**30000**
# of females	30	30	30	30
Females with confirmed copulation /Total paired	100%	93.3%	98.7%	98.7%
Pregnant total/Paired	93.3%	80.0%	80.0%	86.7%
Mean precoital time, days (% of control)	2.84	3.13 (110%)	3.61 (127%)	3.17 (112%)
# of pregnant animals/total number (% of control)	25/30	24/30	23/30	24/30
Males with confirmed copulation/total paired	93.3%	86.7%	83.3%	83.3%
Males impregnating females/total paired	90.0%	73.3%	76.7%	80.0%
Gestational length (days) (% of control)	22.4	22.6 (101%)	22.6 (101%)	22.6 (101%)

Table B.6.6.1/07-07: Mating, fertility and gestation parameters (F1B Second Mating)

	Dietary concentration (ppm)			
	0	**2000**	**10000**	**30000**
# of females	30	30	30	30
Females with confirmed copulation /Total paired	83.3%	83.3%	80.0%	86.7%
Pregnant total/Paired	53.3%	70.0%	83.3%	83.3%
Mean precoital time, days (% of control)	3.69	3.20 (87%)	3.05 (83%)	2.45 (67%)
# of pregnant animals/total number (% of control)	16/30	20/30	19/30	22/30

	Dietary concentration (ppm)			
	0	**2000**	**10000**	**30000**
Males with confirmed copulation/total paired	70.0%	69.0%	70.0%	80.0%
Males impregnating females/total paired	46.7%	58.8%	60.0%	76.7%
Gestational length (days) (% of control)	22.4	22.6 (101%)	22.4 (100%)	22.5 (100%)

LITTER DATA

Size and Viability

Day 0 dead pup counts among treated groups were comparable to the control group for all three litters of pups (F1a, F2a and F2b generation).

A slight reduction in the average litter size (13%) was observed in the F0 dams of the 30000 ppm dose group. This effect was less pronounced in animals after the first F1 mating (10%). Although the difference was not statistically significant and not accompanied by an increase in dead pups/litter, a treatment-related effect could not be excluded. Therefore, a second mating of the F1a adults was performed. In the resulting F2b generation, no dose-related decrease in litter size was observed.

Table B.6.6.1/07-08: Litter size

	Dietary concentration (ppm)			
	0	**2000**	**10000**	**30000**
F0 generation				
# of females	24	29	28	28
Litter size (live)	13.3	12.5	12.7	11.5 (13%)
Dead pups per litter	0.0	0.2	0.1	0.1
F1A (first mating)				
# of females	28	24	24	26
Litter size (live)	12.0	12.3	11.5	10.8 (10%)
Dead pups per litter	0.1	0.3	0.2	0.0
F1A (second mating)				
# of females	16	20	19	25
Litter size (live)	11.9	10.9	13.2	10.7
Dead pups per litter	0.1	0.2	0.2	0.2

Growth and Development

Birth weights and initial growth rate for pups from the treated dams compared well to the ones of the control, except the pups of the 30000 ppm dose group had reduced body weight gains on day 21 of lactation (more than 10% difference to controls). The effect was present earlier in the F1 matings (day 14). This effect was explained by the titrated uptake of the test substance-containing diet at the end of lactation. In the mid dose group, decreases in pups body weight gains were observed compared with the control. These effects were not evident in both sexes from all generations and therefore regarded of questionable toxicological significance. Pup survival during the postnatal period was not affected.

Table B.6.6.1/07-09: Mean pup weights

Dietary concentration (ppm)	**No. of litters[c]**		**Mean group body weight (g) at Day**			
			0	**21**	**0**	**21**
			Males		Females	
			F0 Generation			
0 (Control)	24	mean	6.28	53.39	6.96	50.80
		SD	0.49	3.90	0.52	4.39
2000	29	mean	6.27	51.82	6.91	49.47
		SD	0.48	5.26	0.48	5.05
10000	28	mean	6.43	50.42*	6.15	49.16
		SD	0.47	3.66 (6%)	0.50	3.12

Dietary concentration (ppm)	No. of litters[c]		Mean group body weight (g) at Day			
			0	21	0	21
30000	28	mean	6.47	46.30**	6.12	44.99**
		SD	0.62	4.09 (13%)	0.59	4.34 (11%)
			F1A Generation (First Mating)			
0 (Control)	28	mean	6.33	55.11	5.95	51.93
		SD	0.60	5.64	0.55	5.07
2000	23	mean	6.20	52.47	5.90	51.42
		SD	0.76	9.15	0.70	4.08
10000	22	mean	6.32	51.53*	5.98	48.49*
		SD	0.74	7.35 (6%)	0.64	5.93 (7%)
30000	26	mean	6.50	47.29**	6.05	44.41**
		SD	0.84	4.62 (14%)	0.74	4.90 (14%)
			F1B Generation (Second Mating)			
0 (Control)	16	mean	6.48	55.03	6.04	49.35
		SD	0.75	6.38	0.63	10.96
2000	18	mean	6.17	52.74	5.86	50.73
		SD	0.74	6.12	0.83	5.91
10000	17	mean	6.36	52.29	5.92	49.48
		SD	0.52	3.35	0.47	2.52
30000	24	mean	6.51	44.43**	6.04	43.10**
		SD	0.63	6.86 (19%)	0.55	3.81 (13%)

c: Combined sexes

Table B.6.6.1/07-10: Pup survival data

Dietary concentration (ppm)	No. of litters		Combined (m/f) survival rates (%) for postnatal days		
			0-4	4-14	4-21
			F0 Generation		
0 (Control)	24	mean	96.4	99.0	99.0
		SD	9.64	3.53	3.53
2000	29	mean	97.6	99.6	99.6
		SD	10.41	2.32	2.32
10000	28	mean	99.4*	99.6	99.6
		SD	3.34	2.36	2.36
30000	28	mean	100.0**	98.4	98.4
		SD	0.00	6.65	6.65
			F1A Generation (First Mating)		
0 (Control)	28	mean	96.7	100.0	100.0
		SD	5.77	0.00	0.00
2000	23	mean	90.9	96.7	96.7
		SD	22.62	15.64	15.64
10000	24	mean	96.2	99.5	99.5
		SD	7.20	2.55	2.55
30000	26	mean	99.4	100.0	99.5
		SD	2.18	0.00	2.45
			F1 Generation (Second Mating)		
0 (Control)	18	mean	98.8	100.0	100.0
		SD	2.60	0.00	0.00
2000	19	mean	91.1	99.3	99.3
		SD	24.19	2.95	2.95
10000	19	mean	96.5	94.1	94.1
		SD	12.16	22.96	22.96
30000	23	mean	96.9	100.0	100.0
		SD	11.67	0.00	0.00

Clinical signs
No clinical signs were observed in the offspring of treated animals.

PATHOLOGY
Necropsy
There were no toxicologically significant macroscopic gross lesions attributed to the test chemical administration.

Organ weights
There were no statistically significant organ weight changes, except a slight increase in testes to body weight ratios in F1a adults of the 30000 ppm dose group. This effect was attributed to their lower terminal body weight.

Histopathology
No treatment-related changes were detected.

CONCLUSIONS BY STUDY AUTHOR
Treatment-associated changes were noted in both sexes at the 30000 ppm dose level. A high incidence of soft stools in adults was accompanied by consistent reductions in body weights of adults and pups. Because decreased pup weights occurred later during lactation when pups began supplementing their milk diet with treated food, the cause of the decreased weights in pups may have been due, at least in part, to their consumption of the treated diet. Decreases in pup weights at the 10000 ppm dose level were small, transient and did not occur consistently in both sexes from all generations. Therefore, body weight changes in pups at the middle dose level were considered to be of questionable toxicological significance.

Compared to control, slightly reduced average litter size was noted in F0 dams at 30000 ppm, and to a lesser degree in F1 dams (after the first mating). The differences were not statistically significant and a reduction was not noted when F1 animals were re-mated. Therefore, a relationship to treatment was considered to be equivocal. There was no clear effect on the ability of treated rats to mate, conceive, carry, or deliver offspring. The 10000 ppm dose level was considered the no-observed-adverse-effect-level (NOAEL) for this study.

Assessment and conclusion by applicant:
The NOAEL was considered to be 10000 ppm for adult toxicity for both the F0 and F1 generations (corresponding to 666-711 mg/kg bw/day for males and 777-804 mg/kg bw/day for females). The NOAEL for reproductive toxicity, for both generations and offspring was considered to be 30000 ppm. The NOAEL for developmental toxicity, for both generations and offspring was considered to be 10000 ppm.

Assessment and conclusion by RMS:
In this study, 30 Sprague-Dawley rats/sex/dose group (F0 and F1a generation) were fed daily with glyphosate at concentrations of 2000, 10000 and 30000 ppm through two generations for approximately 11 (F0-generation) and 14 weeks (F1a-generation), respectively. Animals of the F1a generation were mated twice to produce the F2a and F2b-generations. The NOAELs set in previous evaluation RAR (2015) remains.

Treatment was associated with following findings:
- clinical signs (soft stools) observed in adult animals at 30000 ppm
- reduced body weight observed in adult animals at 30000 ppm (Terminal bw: F0 males: 8%, F1 males: 13%, F1 females: 10%; Maternal bw during gestation: Day 1: F0 females: 7%, F1 females first mating: 12%, F1 females second mating: 13%; Day 21: F0 females: 7%, F1 females first mating: 8%, F1 females second mating: 8%)
- reduced litter size (13%) observed in F0 dams at 30000 ppm
- reduced pup weights observed at 30000 ppm (F0 males: 13%, F0 females: 11%; F1A males and females: 14%; F1B males: 19%, F1B females: 13%)

A slight reduction in the average litter size (13%) was observed in the F0 dams of the 30000 ppm dose group, and to a lesser degree in the F1 dams. The reduction was non-statistically significant and not noted when F1 animals were re-mated, and treatment-relation was considered to be equivocal. A reduction in litter size was not

confirmed in other studies where the same strain of animal and similar or higher dose levels were used (*et al*. (2007); *et al*. (1992); (1997).

Decreases in pup weights at the 10000 ppm dose level (666-771 and 777-804 mg/kg bw/day in males and females, respectively) did not occur consistently in both sexes from all generations. Therefore, body weight changes in pups at the middle dose level were considered to be of questionable toxicological significance. This finding at the 10000 ppm level, was not confirmed in the study by et al. (2007), where dose levels up to 15000 ppm (1063 and 1634 mg/kg bw/day) were used or in the study by et al. (1992), where dose level up to 10000 ppm (F0: 668 and 752 mg/kg bw/day in males and females, respectively; F1: 771 and 841 mg/kg bw/day in males and females, respectively) were used. The same strain of rat was used in the studies.

NOAEL for parental toxicity was set at 10000 ppm (666-711 mg/kg bw/day for males and 777-804 mg/kg bw/day for females) based on clinical signs of soft stools and reduced body weight observed in males and females of both generations at 30000 ppm.

NOAEL for offspring was set at 10000 ppm (666-711 mg/kg bw/day for males and 777-804 mg/kg bw/day for females) based on reduced pup weights observed in males and females of both generations at 30000 ppm.

NOAEL for reproductive toxicity was set at 10000 ppm (666-711 mg/kg bw/day for males and 777-804 mg/kg bw/day for females) based on equivocal reduction in litter size observed in F0 dams at 30000 ppm.

The study is acceptable. The study is conducted in accordance with GLP and follows OECD TG 416 (2001) with exception of following deviations, these deviations do not invalidate the study:
(i) minor deviations in housing conditions: Temperature was 18-26ºC (the guideline recommends that the temperature in the experimental animal room should be 22 ± 3°C)
(ii) no data on food efficiency
(iii) no details on fertility indices, number of live births and pre- and post-implantation loss
(iv) no determination of oestrus cycle length
(v) sperm analysis not performed
(vi) no determination of physical or sexual development landmarks
(vii) parental animals: only ovaries and testes with epididymides weighed (uterus, prostate, seminal vesicles, brain, liver, kidneys, spleen, pituitary, thyroid and adrenal glands not weighed)
(viii) brain, spleen and thymus of pups not weighed
(ix) coagulating gland and cervix not included in the histopathological examination
(x) no details on number of pups with grossly visible abnormalities

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- liver weight
- sperm morphology
- sperm motility
- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- prostate weight
- seminal vesicles weight
- uterus weight

Sensitive to but not diagnostic of EATS:

- number of corpora lutea
- number of live births
- pre- and postimplantation loss
- sex ratio
- adrenal weight

B.6.6.1/11

Data point	CA 5.6.1/011
Report author	[redacted]
Report year	1988
Report title	Report on effect of glyphosate technical of [redacted], on fertility and general reproductive performance (Segment I)
Report No.	Not stated
Document No.	42-90619
Guidelines followed in study	No guideline cited in the report and not in accordance with OECD 415 (1983)
Deviations from current test guideline	• test substance purity not given • group size too small • missing endpoints: body weights or food consumption for parental males, body weights for females during premating period and until Day14 of gestation, estrous cycle monitoring, pre-coital interval, duration of gestation, sperm analysis, organ weights, histopathology, monitoring of physical and sexual offspring development, • dose levels tested too low Comments by RMS: The following deviations were observed in addition: • parental animals were dosed 60 days (males) or 14 days (females) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period) • animals were mated in a sex ratio of 1 male:3 females (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating)) • only two dose levels were used (the guideline recommends three dose levels) • a quantitative evaluation of primordial follicles not conducted • statistics not reported Histopathology not performed except for testis.
Previous evaluation	Yes, evaluated and considered supplementary in DAR (1998), not accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory when the study was performed
Acceptability/Reliability	No (See RMS´ assessment below)

MATERIALS AND METHODS

Test material: Glyphosate technical
Lot/Batch: Not reported
Stability of test compound: Not reported
Purity/Radiochemical Not reported

purity:
Vehicle: Corn oil
Animals: Species: Rat
Strain: Wistar rats
Age at start of treatment: Not reported
Sex: Males and females
Initial body weights: 70-130 g (no further information)
Acclimation period: Not reported
Diet/Food: Pelleted food from Lipton India
Water: Not reported
Housing: Polypropylene rat cages with paddy as bedding; single housing during pregnancy, dams were housed with their litters until weaning
Environmental conditions:
Temperature: 19-25 °C
Humidity: 50-70%
Air changes: not reported
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In this reproduction toxicity study groups of 10 male and 30 female Wistar rats received daily doses of 0, 5 and 10 mg/kg bw glyphosate technical per gastric gavage. After approximately 60 days of treatment for males and 14 days of treatment for females pairing of animals within each dose group was undertaken on a one male: three female basis. Males were sacrificed after mating. Pregnant females were either sacrificed on Day 13 of gestation or were allowed to litter and raise their offspring until weaning on Day 21 *post partum*.

Clinical observations
All animals were observed daily for any signs of toxicity.

Body weight
Parental female body weights were recorded periodically during gestation and lactation.

Food consumption
Parental female food consumption was recorded periodically during gestation and lactation.

Mating procedure and vaginal smears
After the scheduled period of treatment (approx. 60 days for males and 14 days for females), pairing of animals within each dose group was undertaken on a one male: three female basis. Successful mating (Day 0 of pregnancy) was confirmed by presence of vaginal plugs.

Pre-coital interval
Not reported.

Duration of gestation
Not reported.

Reproduction parameters
Half of the females were sacrificed on Day 13 of gestation and the uteri were examined for living foetuses, early and late deaths and the ovaries for the number of corpora lutea. The remaining females were allowed to litter and rear their offspring until day 21 *post partum*.

Litter data
Litter size, growth rate and survival rates of pups were recorded.

Assessment of development and reproductive performance of progeny
Not reported.

Sacrifice and pathology
Males were sacrificed after mating. Pregnant females were either sacrificed on Day 13 of gestation or at weaning on Day 21 post partum.

Statistics
Not reported.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS
Not reported.

MORTALITY
There were no mortalities in parental animals.

CLINICAL OBSERVATIONS
No clinical signs of toxicity were noted throughout the study.

BODY WEIGHT
There were no effects on body weights in female rats up to and including 10 mg glyphosate/kg bw/day.

Table B.6.6.1/08-01: Body weights (group mean) of females

Body weight (g) ± SE	**Dose level (mg/kg bw/day)**		
Day	**0 (control)**	**5**	**10**
Post coitum			
15	210.5 ± 5.65	194.2 ±4.47	200.7 ± 4.06
20	225.4 ± 5.07	211.3 ±4.81	217.2 ± 4.17
Post partum			
0	172.6 ± 5.33	174.7 ± 5.10	166.2 ± 4.25
6	170.6 ± 4.90	179.2 ± 5.10	168.5 ± 4.31
12	172.9 ± 5.00	179.3 ± 4.99	171.2 ± 4.02
18	174.8 ± 4.80	180.3 ± 4.62	171.5 ± 3.87
21	176.5 ± 5.16	180.7 ± 4.51	172.2 ± 3.77

FOOD CONSUMPTION
There were no effects on food consumption in female rats up to and including 10 mg glyphosate/kg bw/day.

REPRODUCTIVE PARAMETERS
When the animals were sacrificed on Day 13 of gestation, the number of corpora lutea, total implantations, early and late deaths and living implantations were similar in test item-treated and control animals.

When allowed to give birth, the litter size, growth and survival rate of test item-treated and control animals were similar.

Table B.6.6.1/08-02: Reproduction data Day 13 of gestation

Parameter	**Dose level (mg/kg bw/day)**		
	0 (control)	**5**	**10**
No. of dams in group	13	12	12
Total No. of corpora lutea	113	107	121
Total No. of implantations	108	104	117
Total No. of early deaths	3	1	1
Total No. of late deaths	2	1	2
Total No. of living foetuses	103	102	114

LITTER DATA

Number of pups delivered

There was no relevant difference in litter size between the three groups.

Viability index

A small number of dead pups was noted between *post partum* days 0 and 4 in all dose groups including the control. The incidence of dead pups was not affected by treatment with the test item. No pup mortality was noted in any group from Day 4 post partum until weaning on Day 21 *post partum*.

Table B.6.6.1/08-03: Litter size and survival rates (group means)

Parameter	Dose level (mg/kg bw/day)		
	0 (control)	**5**	**10**
Mean litter size ± SE	8.92 ± 0.73	8.92 ± 0.54	8.58 ± 0.66
Survival rates (%)			
Day 0	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
Day 4	97.3 ±1.51	98.4 ± 1.03	99.2 ± 0.76
Day 14	97.3 ± 1.51	98.4 ± 1.03	99.2 ± 0.76
Day 21	97.3 ± 1.51	98.4 ± 1.03	99.2 ± 0.76

Body weights

Pup weights generally developed similar in the test item-treated groups and the control group.

Table B.6.6.1/08-04: Pup body weights (group means)

Body weight (g) ± SE		Dose level (mg/kg bw/day)		
Day		**0 (control)**	**5**	**10**
Post partum				
0		5.77 ± 0.10	5.42 ± 0.14	5.70 ± 0.19
4		10.09 ± 0.24	9.41 ± 0.28	9.97 ± 0.41
14		18.87 ± 0.59	17.96 ± 0.48	19.76 ± 1.02
21		29.56 ± 0.69	29.25 ± 0.55	30.37 ± 0.62

HISTOPATHOLOGY OF TESTIS

No histopathological findings were noted in the testes of any animal on study.

CONCLUSION BY STUDY AUTHOR

Glyphosate technical supplied by [redacted], did not produce any toxic effect on fertility and general reproductive performance up to the high dose of 10 mg/kg bw/day.

Assessment and conclusion by applicant:

Under the conditions of this study, glyphosate technical did not produce any effects on fertility and general reproductive performance up to the high dose of 10 mg/kg bw/day.

Assessment and conclusion by RMS:

Groups of rats were administered glyphosate technical orally, by gavage, at dose levels of 0, 5 and 10 mg/kg bw/day. Controls received the vehicle (corn oil), only. Male rats were treated for 60 days before mating while treatment of female rats started 14 days before mating and was continued during gestation and lactation.
No treatment-related effects were observed in the study.

The study is not acceptable (dose levels tested much too low and other major deviations from the OECD TG 416 (2001)).

Following deviations from the OECD TG 416 (2001) were observed:
(i) purity not specified

(ii) group size too small (12-13 dams/group) (the guideline recommends at least 20 dams/group)
(iii) parental animals were dosed 60 days (males) or 14 days (females) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period)
(iv) animals were mated in a sex ratio of 1 male:3 females (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating))
(v) only two dose levels were used (the guideline recommends three dose levels)
(vi) dose levels tested too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering)
(vii) body weights for females during premating period and until Day14 of gestation missing
(viii) oestrous cycle monitoring not performed
(ix) pre-coital interval not recorded
(x) duration of gestation not evaluated
(xi) no sperm analyses
(xii) a quantitative evaluation of primordial follicles not conducted
(xiii) sexual maturation not investigated
(xiv) no organ weighed
(xv) histopathology not performed except for testis
(xvi) statistics not reported

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

<u>EATS-mediated parameters not investigated:</u>

- thyroid weight
- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- sperm morphology
- sperm motility
- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- epididymis histopathology
- epididymis weight
- liver weight
- ovary histopathology
- ovary weight
- prostate histopathology
- prostate weight
- seminal vesicles histopathology
- seminal vesicles weight
- testis weight
- uterus histopathology
- uterus weight
- vagina histopathology

<u>Sensitive to but not diagnostic of EATS:</u>

- adrenal weight
- gestation length
- pituitary weight
- presence of anomalies
- sex ratio

- time to mating

B.6.6.1/12

Data point	CA 5.6.1/012
Report author	[redacted]
Report year	1988
Report title	Report on effect of pesticides on reproductive process - Segment IV - Three generation reproduction study with alblino rats using glyphosate technical of [redacted]
Report No.	Not reported
Document No.	Not reported
Guidelines followed in study	No guideline cited in the report and not in accordance with OECD 416 (1983 or 2001)
Deviations from current test guideline	Test substance purity not given; Group size too small; no justification for dose levels; no information on pre-mating dosing period; missing endpoints: estrous cycle monitoring, pre-coital interval, duration of gestation, body weights during gestation, sperm analysis, organ weights, monitoring of physical and sexual offspring development Comments by RMS: The following deviations were observed in addition: • parental animals were dosed 60 days (males) or 14 days (females) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period) • animals were mated in a sex ratio of 1 male:2 females to produce the F1 litters (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating)) • age of F0 animals at initiating of dosing was 14 weeks (the guideline recommends the animals to be 5 to 9 weeks old at the start of dosing) • dose levels tested too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering) • a quantitative evaluation of primordial follicles not conducted • histopathology limited (vagina, cervix, epididymides, prostate, coagulating gland not included in the histopathological examination) • statistics not reported
Previous evaluation	Yes, evaluated and considered supplementary in DAR (1998), not accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory when the study was performed
Acceptability/Reliability	No (See RMS´ assessment below)

MATERIALS AND METHODS

Test material:	Glyphosate technical
Lot/Batch:	Not reported Stability of test compound: Not reported
Purity/Radiochemical purity:	Not reported
Vehicle:	Plain diet
Animals:	Species: Rat Strain: Wistar rats Age at start of treatment: 100 days (F0 generation)

Sex: Males and females
Mean body weight at initiation of dosing: Males: 148 - 150 g; females: 137 - 144 g
Acclimation period: Not reported
Diet/Food: Pelleted food from Lipton India, Bangalore
Water: *ad libitum*
Housing: Polypropylene rat cages with paddy as bedding; single housing during pregnancy, dams were housed with their litters until weaning
Environmental conditions:
Temperature: 19-25 °C
Humidity: 30-70%
Air changes: not reported
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In this three-generation reproduction study groups of 8 male and 16 female Wistar rats of the F0 generation received daily dietary doses of 0, 75, 150 and 300 ppm glyphosate technical in diet (using a conversion factor of 20, this concentration would correspond to an approximate daily intake of 0, 3.75, 7.5 and 15 mg/kg bw/day). Animals were kept on this diet until weaning of the F3b generation.

After 60 days and 14 days of treatment for males and females, respectively, pairing of animals mating within each dose group was undertaken on a one male: two female basis, to produce the F1 litters. Pregnant females were allowed to deliver and rear their litters (F1a) until weaning on Day 21 *post partum*. After a rest of 10-15 days parental females were mated again with the same male in a similar manner to deliver and rear the F1b litter. Eight males and 16 females were chosen from the F1b offspring as parents for the F2 generation. The mating of siblings was avoided. The procedure described above was repeated to produce the F3 offspring. Standardisation of litter size: on day 4 *post partum* litter size was adjusted to 4 males and 4 females, where possible.

Diet preparation and analyses
The test item was mixed with food to achieve concentrations of 75, 150 and 300 ppm. The control group received plain food.

Clinical observations
A gross general observation was made for each animal once a day.

Body weight
Body weights of F0 animals, and for F1 and F2 animals selected for rearing were recorded weekly. Body weights of the F0 animals were recorded during mating and weaning period only. Offspring not selected to produce a next generation was weighed on days 0, 4, 14 and 21 *post partum*. F1 offspring (males) selected to produce the F2 generation were weighed up to and during mating. F2 offspring (males) selected to produce the F3 generation were weighed up to and during mating for the F3b generation. F1 offspring (females) selected to produce the F2 generation were weighed up to weaning of the F2b generation. F2 offspring (females) selected to produce the F3 generation were weighed up to weaning of the F3b generation.

Food consumption and compound intake
The amount of food consumed was determined once weekly for parental animals of all three generations. However, reported values do not include data for all animals in every group.

Mating procedure and vaginal smears
After the scheduled period of treatment (60 days for males and 14 days for females), females were paired on a one- to-two basis with males from the same treatrnent group for a maximum of one week.

Pre-coital interval
Not reported.

Duration of gestation
Not reported.

Reproduction parameters
For each generation and mating phase, the number of dams mated, pregnant and littering were recorded.

Litter data
For each generation and mating phase, the number of live and dead pups per dam were recorded.

Assessment of development and reproductive performance of progeny
Not reported.

Sacrifice and pathology
All F1a offspring was sacrificed after weaning. F0 males were sacrificed after mating for the F1b generation. Necropsy was performed and testes were preserved for histopathological examination.

F1b offspring not selected for rearing were sacrificed after weaning. All F2a offspring were sacrificed after weaning. F1b parental males were sacrificed 1 week after mating for the F2b generation. Necropsy was performed and a range of organs (see below) including testes were preserved for histopathological examination. F2b offspring not selected for rearing was sacrificed after weaning. F1b and F2b parental females were sacrificed after weaning of the F2b and F3b generation, respectively. Necropsy was performed and the following tissues were preserved for histopathological examination: adrenals, aorta, brain, colon, duodenum, eyes, heart, ileum, kidney, liver, lung, oesophagus, ovaries, pancreas, pituitary, seminal vesicles, spleen, stomach, testes, thyroid, urinary bladder, uterus and salivary glands. All offspring of the F3a generation was sacrificed after weaning. F2b parental males were sacrificed 1 week after mating for the F3b generation. Necropsy was performed and the tissue listed above were preserved for histopathological examination. All F3b offspring was sacrificed after weaning.

Statistics
Not reported.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE CONCENTRATION IN THE DIET
Not reported.

TEST COMPOUND INTAKE
Not reported.

MORTALITY
There were no mortalities in animals after weaning. A low incidence of pup mortality was noted in all groups without any test item effects.

CLINICAL OBSERVATIONS
No clinical signs of toxicity were noted throughout the study.

BODY WEIGHT
There was no substantial difference in the body weight gain of test item-treated animals at any dose level compared to the respective control animals.

FOOD CONSUMPTION
No significant differences in food consumption were observed between test item-treated and control animals in any of the three generations.

REPRODUCTIVE PARAMETERS
Pregnancy rate
The number of pregnant females was not significantly different between test item-treated and control groups.

Table B.6.6.1/09-01: Reproduction data F0 animals

	Group 1 - control		Group 2		Group 3		Group 4	
	0 ppm		75 ppm		150 ppm		300 ppm	
	F1a	F1b	F1a	F1b	F1a	F1b	F1a	F1b
Number of dams in group	16	12	16	13	16	12	16	12
Number of dams mated	16	12	16	13	16	12	16	12
Number of dams pregnant	12	9	13	9	12	9	12	9
Number of dams littered	12	9	13	9	12	9	12	9
Mean litter size ± SE	9.0 ± 0.28	8.9 ± 0.35	8.6 ± 0.29	9.0 ± 0.29	8.8 ± 0.37	8.7 ± 0.33	8.8 ± 0.33	9.0 ± 0.29
Mean number of live pups/litter ± SE	8.8 ± 0.25	8.6 ± 0.29	8.4 ± 0.24	8.7 ± 0.24	8.4 ± 0.29	8.3 ± 0.29	8.4 ± 0.29	8.7 ± 0.33
Mean number of dead pups/litter ± SE	0.25 ± 0.13	0.33 ± 0.17	0.23 ± 0.12	0.33 ± 0.17	0.33 ± 0.14	0.33 ± 0.17	0.33 ± 0.14	0.33 ± 0.17

Table B.6.6.1/09-02: Reproduction data F1 animals

	Group 1 - control		Group 2		Group 3		Group 4	
	0 ppm		75 ppm		150 ppm		300 ppm	
	F2a	F2b	F2a	F2b	F2a	F2b	F2a	F2b
Number of dams in group	16	12	16	12	16	13	16	12
Number of dams mated	16	12	16	12	16	13	16	12
Number of dams pregnant	12	9	12	10	13	10	12	9
Number of dams littered	12	9	12	10	13	10	12	9
Mean litter size ± SE	8.7 ± 0.31	8.7 ± 0.41	8.8 ± 0.30	8.4 ± 0.34	8.4 ± 0.24	8.9 ± 0.23	8.4 ± 0.29	8.7 ± 0.24
Mean number of live pups/litter ± SE	8.4 ± 0.31	8.3 ± 0.33	8.4 ± 0.29	8.1 ± 0.23	8.1 ± 0.21	8.6 ± 0.22	8.2 ± 0.21	8.3 ± 0.24
Mean number of dead pups/litter ± SE	0.25 ± 0.13	0.33 ± 0.24	0.33 ± 0.14	0.30 ± 0.15	0.31 ± 0.17	0.30 ± 0.15	0.25 ± 0.18	0.30 ± 0.17

Table B.6.6.1/09-03: Reproduction data F2 animals

	Group 1 - control		Group 2		Group 3		Group 4	
	0 ppm		75 ppm		150 ppm		300 ppm	
	F3a	F3b	F3a	F3b	F3a	F3b	F3a	F3b
Number of dams in group	16	13	16	12	16	13	16	13
Number of dams mated	16	13	16	12	16	13	16	13
Number of dams pregnant	13	10	12	9	13	10	13	10
Number of dams littered	13	10	12	9	13	10	13	10
Mean litter size ± SE	8.2 ± 0.30	8.5 ± 0.34	8.4 ± 0.31	8.8 ±0.28	8.3 ± 0.29	8.8 ± 0.25	8.5 ± 0.32	8.7 ± 0.37
Mean number of live pups/litter ± SE	7.9 ± 0.19	8.2 ± 0.25	8.2 ± 0.24	8.4 ± 0.24	8.1 ± 0.27	8.5 ± 0.27	8.2 ± 0.27	8.4 ± 0.34
Mean number of dead pups/litter ± SE	0.31 ± 0.18	0.30 ± 0.15	0.25 ± 0.13	0.33 ± 017	0.23 ± 0.12	0.30 ± 0.15	0.31 ± 0.13	0.30 ± 0.15

LITTER DATA

Number of pups delivered

Litter size varied from 7 to 10 in all four groups in all three generations and there was no significant difference in litter size.

Viability index
The number of dead pups per litter varied from 0 to 2 and the rate was less than 3.3 pups per litter. There was no significant difference in the number of dead pups in the test item-treated groups when compared to their respective control group.

Body weights
Pup weights generally developed similar in the test item-treated groups and the respective control groups.

Histopathology
Histopathological examination of various tissues from animals at all dose levels did not reveal any structural changes attributable to treatment with the test item.

CONCLUSION BY STUDY AUTHOR
There were no adverse effects of treatment observed in any of the parameters investigated. Thus, a dose level of 300 ppm was considered the NOEL for reproduction toxicity.

Assessment and conclusion by applicant:
supportiveThere were no adverse effects of treatment observed in any of the parameters investigated in this study, thus a dose level of 300 ppm was considered the NOEL for both parental and reproduction toxicity. Using the usual conversion factor of 20, this concentration would correspond to an approximate daily intake of 15 mg/kg bw/day.

Assessment and conclusion by RMS:
Glyphosate technical was given via the diet at dose levels of 0, 75 and 150 or 300 ppm to Wistar rats over three generations. In the F0 generation, the groups consisted of 8 male and 16 female animals from which similar groups were produced by selective mating for the F1, F2 and F3 generations. Two litters per generation were delivered. The rats were observed for changes in body weight and food consumption, pregnancy rate, litter size, number of live and stillborn pups and any abnormalities. Testes of F0 males and selected organs and tissues of parental animals in the F1 and F2 generations were examined histopathological. There were no adverse effects of treatment observed in any of the parameters investigated.

The study is not acceptable (dose levels tested much too low and other major deviations from the OECD TG 416 (2001).

Following deviations from the OECD TG 416 (2001) were observed:
(i) purity not specified
(ii) parental animals were dosed 60 days (males) or 14 days (females) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period)
(iii) animals were mated in a sex ratio of 1 male:2 females to produce the F1 litters (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating))
(iv) Age of F0 animals at initiating of dosing was 14 weeks (the guideline recommends the animals to be 5 to 9 weeks old at the start of dosing)
(v) group size too small (12-16 dams/group) (the guideline recommends at least 20 dams/group)
(vi) dose levels tested too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering)
(vii) no information on pre-mating dosing period
(viii) body weights during gestation not recorded
(ix) oestrous cycle monitoring not performed
(x) pre-coital interval not recorded
(xi) duration of gestation not evaluated
(xii) no sperm analyses
(xiii) a quantitative evaluation of primordial follicles not conducted
(xiv) physical and sexual maturation not investigated in offsprings
(xv) no organ weighed
(xvi) vagina, cervix, epididymides, prostate, coagulating gland not included in the histopathological examination for adults

(xvii) statistics not reported

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- histopathological changes of the thyroid glands (considered to be optional according to EFSA guidance document (EFSA Journal 2018; 16(6):5311)
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- sperm morphology
- sperm motility
- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- epididymis histopathology
- epididymis weight
- liver weight
- ovary weight
- prostate histopathology
- prostate weight
- seminal vesicles weight
- testis weight
- uterus weight
- vagina histopathology
- vaginal smears

Sensitive to but not diagnostic of EATS:

- adrenal weight
- gestation length
- pituitary weight
- presence of anomalies
- sex ratio
- time to mating

B.6.6.1/13

Data point	CA 5.6.1/013
Report author	[redacted]
Report year	1985
Report title	Three-generation reproduction study in rats with the oral administration of glyphosate
Report No.	Not reported
Document No.	OX9650161
Guidelines followed in study	None (pre-guideline)
Deviations from current test guideline	No details on the test material (batch and purity) were provided; no analytical determinations on stability and homogeneity of the test material in the diet; no justification for the selection of dose levels was given; the top dose level of the present study (5000 ppm, corresponding to approx. 500 mg/kg bw/day) was rather low; no data on individual animals or summarised data were provided on clinical signs of toxicity and mortality of adult animals; statistical analysis was only conducted for some of the parameters

	investigated and not always indicated in result tables; the starting body weight of F0+ animals was statistically significantly higher for glyphosate-treated animals than for control animals; a limited set of parameters for haematology and clinical chemistry was investigated; the oestrus cycle of the animals was not monitored and no information on time to mating or confirmation of pregnancy was reported; no absolute but only relative organ weights were reported; no historical control data included in the study report. Comments by RMS: The following deviations were observed in addition: • mating period was 6 consecutive days (the guideline recommends a 2-week mating period) • during mating, males changed daily so that each female cohabited with different males (the guideline recommends for each mating each female should be placed with a single male from the same dose level (1:1 mate) until copulation occurs 2 weeks have elapsed). • age of F0 animals at initiating of dosing was 28 days (the guideline recommends the animals to be 5 to 9 weeks old at the start of dosing) • few animals were used. Six F0 animals/sex/dose group were used for production of the F1 generations, F1A and F1B generations. Twelve animals/sex/dose groups of the F1B generation were mated to get F2 generations (the guideline recommends each test and control group should contain a sufficient number of animals to yield preferable not less than 20 pregnant females at or near parturition) • pre-coital interval not recorded • no sperm analyses • a quantitative evaluation of primordial follicles not conducted • sexual maturation not investigated in offsprings • uterus, ovaries, seminal vesicles with coagulating glands, pituitary and thyroid not weighed • vagina, uterus with cervix, ovaries, testis, epididymidis, prostate, seminal vesicles, coagulating gland not included in the histopathological examination for adults
Previous evaluation	Yes, evaluated and accepted in DAR (1998), not accepted in RAR (2015)
GLP/Officially recognised testing facilities	No, GLP was not compulsory when the study was performed
Acceptability/Reliability	Not acceptable (large number of deviations and reporting deficiencies)

MATERIALS AND METHODS

Test material: Glyphosate
Chemical name: N-/phosphonomethyl/glycine
Lot/Batch: Not reported
Stability of test compound: Not reported
Expire date: Not reported
Purity/Radiochemical purity: Not reported
Vehicle:
Animals: Species: Rat
Strain: Wistar MD
Source: [redacted]
Number of animals (F0 generation): 128 (64 males and 64 females)
Age at start of treatment: 28 days
Sex: Males and females
Initial body weights: 230-265 g (males) and 145-170 g (females)
Acclimation period: 2 weeks
Diet/Food: Standard diet (LATI)

Water: Not reported
Housing: Prior to mating, the animals were kept in groups of 5/sex in Makrolon type II cages with Wood shavings as bedding. After mating the females were housed individually during their preganancy and lactation period.
Wood shavings, changed daily
Temperature: 21±º C
Humidity: 55-70%
Air changes: 8/hour
12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
The test item was incorporated into the diet and administered to three groups of 16 male and 16 female rats of the F0 generation (F0+ and F0). Dose levels were 200, 1000 and 5000 ppm, corresponding to mean achieved doses of 17.8, 92.2 and 462.2 mg/kg bw/day in males and 19.2, 106.0 and 502.0 mg/kg bw/day in females. A similar constituted group of 16 control animals per sex received the plain diet and served as control. After 12 weeks of dietary administration, 10/16 rats/sex (F0+ animals) were sacrificed from each dose group and examined for haematology, serum chemistry, organ weights, gross necropsy and histopathology.

The remaining 6 rats/sex/dose group from the F0 generation (F0 animals) were mated to form the F1 generations, F1A and F1B. Mating was conducted for 6 consecutive days: Males were changed daily, so that each of the 6 females was cohabitated with 6 different males.

Twelve animals/sex/dose groups of the F1B generation were mated to get F2 generations while F1A offspring were sacrifice on day 28. Similarly, F2A offspring were sacrificed on day 28, while 24 animals/sex/group of F2B generation were mated to get the F3 generation.

F3A and F3B offspring were sacrificed at weaning, while 10 animals/sex/dose levels of the F3C offspring were treated for 8 weeks and then received untreated diet for 4 weeks of recovery period.

Table B.6.6.1/10-01: Animal assignment and fate

Generation	Group	No. of animals tested /sex /dose group	Fate
F0	F0+	10	Baseline animals for assessing systemic toxicity.
	F0	6	Animals for production of the F1 generation
F1	F1A	None	Animals for observation of developmental effects in F1 generation
	F1B	12	Animals for observation of developmental effects in F1 generation and for production of the F2 generation
F2	F2A	None	Animals for observation of developmental effects in F2 generation
	F2B	24	Animals for observation of developmental effects in F2 generation and for production of the F3 generation
F3	F3A	None	Animals for observation of developmental effects in F3 generation#
	F3B	None	Animals for observation of developmental effects in F3 generation#
	F3C	10	Animals for observation of developmental effects in F3 generation and for investigation of recovery after treatment

Diet preparation and analyses
For each dose level, a single batch of glyphosate in the diet was prepared. For preparation of diet mixtures, appropriate quantities of glyphosate were mixed into standard food sizing. The mixture was homogenised in the lack of water first, later with water addition of 6%. Pellets of 10 x 30 x 40 cm were prepared with the help of a Simon-Heesen condenser. Batches of approx. 10 kg were stored at -20°C until usage.
The stability and homogeneity of the test material in the diet were not determined.

Clinical observations
All animals were observed daily (6 times/week) for their general condition and mortality/ moribundity. At weekly intervals (in parallel to body weight measurement), behaviour and appearance of the animals were assessed.

Food consumption
Food consumption (based on unconsumed pelletised food) was measured at weekly intervals for all adult animals. Re-measuring was done after a consuming period of 48 hours. For each animal, the amount of food consumption in g/kg/week was calculated. The actual test compound intake in mg/ kg bw/week and compound intake in mg/kg bw/day were calculated from the actual food consumption.

Haematology and clinical chemistry
Haematology and clinical chemistry was performed for all adult animals. Blood was collected from fasted animals prior to sacrifice under ether anaesthesia. All blood available in the opened abdominal cavity of the animal was collected into tubes with and without heparin. Heparinised blood was used for cell counting, all other tests were performed from the native blood.

For haematological examination, the following parameters were evaluated: haemoglobin*, haematocrit, erythrocytes, leukocytes, thrombocytes, reticulocytes, coagulation time, segment, eosinophil, basophil, lymphocytes and monocytes.

* Haemoglobin levels were listed under „Clinical chemistry“ parameters in the study report. For evaluation in this OECD summary, the parameter was shifted to “Haematology”.

For clinical chemistry analysis the following parameters measured: glucose, urea, carbamide, total bilirubin, total protein, total lipids, cholesterol, triglycerides, alkaline phosphatase, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, lactate dehydrogenase, cholinesterase, sodium, potassium and calcium.

Reproduction parameters
The following parameters were investigated to monitor reproduction: reproductive performance, conception, birth rate and maternal behaviour. In addition, the fertility index was recorded for each group at each generation.
Oestrus cycle and length were not evaluated in the females of any generation. Sperm parameters were examined by organ weight analysis and histopathology of testicles and epididymides. For details, please refer to the section “Sacrifice and pathology”.

Litter data
Pups of all generations were examined for behaviour, general appearance and mortality. On Days 0, 4, 7, 14, 21 and 28 the offspring was counted to record the number of live and dead male and female pups. The development of the foetuses was monitored by determination of body weight and body weight gain (termed “growth rate” in study report) on Days 0, 4, 7, 14, 21 and 28.

During the weaning period (lactation Days 1 – 28), the pups were observed for any morphological and terato-behavioural changes. For each dose group, the following indices determined: vitality index, lactation index and postnatal survival index. In addition, the sex ratio was determined for each generation (including separation for A and B).

Sacrifice and pathology
At scheduled sacrifice, macroscopic examinations were performed after the 12-week dosing period (F0+, F3C generations) and after weaning (F0, F1B and F2B generations).
No details on the list of organs examined was provided.

Organ weights
From all adult animals, the following organ weights were collected at scheduled necropsy: brain, heart, thymus, stomach, spleen, lung, liver, kidney, adrenal, testicles and epididymides. All weights recorded were reported as relative organ weights to brain weight.

Histopathology
Histopathology was performed on adult animals of all treatment groups. 3 rats/sex were examined of the F0+ (after 12 weeks of treatment) and F0 animals (after weaning of the F1 generation). Each 5 rats/sex/dose level were examined from the F1B (after weaning of the F2 generation), F2B (after weaning of the F3 generation) and F3C generations (after 8 weeks of treatment followed by 4 weeks of recovery). The following organs and tissues were collected: brain, pituitary, thyroid, parathyroid, oesophagus, trachea, thymus, heart, lung, stomach, liver, duodenum, pancreas, spleen, abdominal lymphnode, kidney, adrenal, testicle, epididymides and ovary. The organs and tissues collected were fixed in Carnoy and Susa fixation, embedded in paraffin wax, sectioned at 5 – 10 µm and stained with haematoxylin-eosin. For the following organs, different staining techniques were applied: Pituitary (aniline blue eosin), lymphnodes (toluidine blue eosin) and liver (periodic acid-fuchsin and Sudan IV).

Statistics
The Double T-Students Test was used for the evaluation of the body and organ weights, for the evaluation of haematology and clinical pathology data. Data of the treated groups were compared to the data of the untreated controls.

RESULTS

ANALYSIS OF DOSE FORMULATIONS
Analytical determinations on the stability and homogeneity of the test item in the diet were not performed.

MORTALITY
For the adult animals of the F0/F0+, F1B, F2B and F3C generations, no incidences of mortality were reported. Mortality observed for the offspring is described under “Litter data”.

CLINICAL OBSERVATIONS
The animals of all generations were reported to show a normal appearance and behaviour throughout the whole study and treatment period. Individual clinical signs of toxicity were not reported.

BODY WEIGHT
Prior to the start of treatment, the absolute body weight of the high dose group (5000 ppm) F0+ males was about 10 g (+8%) higher than for the remaining F0+ males of the other dose groups. The statistically significant difference in body weight existed throughout the whole treatment period (+8% at the end of treatment). Statistically significant increases in absolute body weight was also observed for F0+ males at 200 ppm at the beginning of treatment, but the difference was only marginal (+3%) and disappeared over the 12-weeks treatment period. For remaining dose groups of the F0+/F0 generation and for the animals of the F1, F2 and F3 generation, body weights were comparable among animals of the same sex at the start of treatment. Due to the generally high body weight of F0+ males prior to treatment and in the absence of findings in other generations, the finding in the F0+ males was not considered as an adverse, treatment-related effect.

For F0+ females, a slight but statistically significant decrease in absolute body weight was further observed at 200 ppm during treatment weeks 8 – 10. The effect was of minor magnitude (-4.4%), transient and not dose-related and therefore considered to be incidental. There were no further changes on absolute body weight noted for males and females of any other generation.

Throughout the whole treatment period, the body weight gain of the animals of all dose groups was comparable to those of control animals for all generations. This observation was the same for F3C animals during the 8 weeks of treatment and 4 weeks of recovery. Thus, body weight and body weight gain were considered unaffected by treatment over all generations. For details, please refer to the table below.

Table B.6.6.1/10-02: Findings in body weight development (mean ± standard deviation)

Dose group	Males				Females			
(ppm)	0	200	1000	5000	0	200	1000	5000
Body weight in F0+ animals								
Week 0	235 ± 6	243 ± 9*	238 ± 5	254 ± 8***	154 ± 7	151 ± 5	155 ± 8	156 ± 6

Dose group	Males				Females			
(ppm)	0	200	1000	5000	0	200	1000	5000
Week 4	298 ± 16	317 ± 19*	309 ± 17	322 ± 18**	182 ± 8	180 ± 10	183 ± 10	186 ± 8
Week 8	343 ± 22	366 ± 26*	358 ± 25	371 ± 21**	204 ± 10	195 ± 7*	204 ± 12	207 ± 11
Week 12	375 ± 25	400 ± 29	395 ± 34	406 ± 24*	218 ± 11	211 ± 8	217 ± 14	222 ± 12
BWG total	141 ± 23	158 ± 25	158 ± 33	152 ± 21	65 ± 10	60 ± 8	63 ± 10	66 ± 13
Body weight in F0 animals								
Week 0	238 ± 8	238 ± 6	244 ± 9	242 ± 9	159 ± 4	157 ± 5	154 ± 5	155 ± 4
Week 4	303 ± 19	302 ± 19	316 ± 20	307 ± 10	184 ± 12	188 ± 9	183 ± 11	181 ± 12
Week 8	356 ± 23	343 ± 24	362 ± 24	358 ± 17	204 ± 11	205 ± 9	204 ± 14	203 ± 14
Week 12	389 ± 31	373 ± 40	396 ± 24	396 ± 22	222 ± 21	227 ± 11	218 ± 18	220 ± 14
BWG total	151 ± 26	134 ± 45	152 ± 18	154 ± 25	63 ± 18	70 ± 8	64 ± 14	65 ± 10
Body weight in F1B animals								
Week 0	131 ± 11	122 ± 13	128 ± 20	129 ± 13	113 ± 12	108 ± 8	106 ± 13	108 ± 9
Week 4	274 ± 21	275 ± 17	272 ± 36	271 ± 18	186 ± 12	184 ± 14	181 ± 17	181 ± 13
Week 8	350 ± 29	351 ± 28	350 ± 51	347 ± 28	219 ± 14	215 ± 16	216 ± 17	213 ± 16
Week 12	388 ± 36	398 ± 34	394 ± 56	392 ± 32	234 ± 13	232 ± 17	231 ± 16	229 ± 17
BWG total	257 ± 29	275 ± 33	266 ± 43	263 ± 26	121 ± 12	124 ± 16	125 ± 11	121 ± 14
Body weight in F2B animals								
Week 0	90 ± 13	89 ± 12	94 ± 14	90 ± 10	82 ± 10	81 ± 11	83 ± 16	83 ± 8
Week 4	234 ± 20	235 ± 21	239 ± 24	237 ± 18	163 ± 10	164 ± 11	164 ± 21	167 ± 10
Week 8	313 ± 27	316 ± 25	319 ± 28	318 ± 25	199 ± 11	200 ± 15	202 ± 24	206 ± 13
Week 12	358 ± 32	361 ± 30	365 ± 33	367 ± 31	216 ± 12	216 ± 17	220 ± 24	224 ± 15
BWG total	268 ± 28	273 ± 24	271 ± 24	277 ± 28	135 ± 10	135 ± 16	137 ± 10	141 ± 15
Body weight in F3C animals								
Week 0	110 ± 14	97 ± 13	97 ± 10	99 ± 9	96 ± 11	89 ± 14	91 ± 8	91 ± 9
Week 4	244 ± 17	234 ± 22	232 ± 20	235 ± 20	168 ± 10	168 ± 21	165 ± 14	171 ± 9
Week 8	330 ± 20	322 ± 30	320 ± 28	327 ± 22	207 ± 19	207 ± 24	211 ± 17	211 ± 10
Week 12#	367 ± 25	360 ± 37	359 ± 30	368 ± 26	220 ± 20	223 ± 22	223 ± 8	228 ± 12
BWG total	257 ± 24	263 ± 30	262 ± 26	270 ± 23	123 ± 20	134 ± 14	133 ± 12	137 ± 11

BWG total: total body weight gain from Weeks 0 - 12 of treatment; *, ** and ***: statistically significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***); #: following 8 weeks of treatment, the animals received plain diet for 4 additional weeks

FOOD CONSUMPTION AND COMPOUND INTAKE

There were no statistically significant effects on food consumption in male or female rats of any generation throughout the 12-week treatment periods.

In general, food consumption was higher at the beginning of treatment than at the end of treatment. In F1B males and females, the food consumption of all treatment and control animals was lower in dosing Week 5 than before or thereafter, but increased again and was as expected until the end of the treatment period.

The mean achieved compound intake over the 12 weeks treatment period was comparable for all generations. Over all generations, mean achieved dose levels at 200, 1000 and 5000 ppm were 17.8, 92.2 and 462.2 mg/kg bw/day in males and 19.2, 106.0 and 502.0 mg/kg bw/day in females.

Table B.6.6.1/10-03: Findings in food consumption (mean ± standard deviation) and mean achieved compound intake

Dose group	Males				Females			
(ppm)	0	200	1000	5000	0	200	1000	5000
F0+ generation: FC over 12 weeks of dietary administration								

Dose group	Males				Females			
(ppm)	0	200	1000	5000	0	200	1000	5000
FC (g/kg animal/week)	503 ± 74	493 ± 81	506 ± 82	505 ± 80	596 ± 103	594 ± 120	582 ± 123	594 ± 120
CI (mg/kg bw/day)	-	14	72	360	-	17	83	424
F0 generation: FC over 12 weeks of dietary administration prior to mating, no treatment during gestation up to lactation Day 28 of the F1 generation								
FC (g/kg animal/week)	513 ± 83	469 ± 79	512 ± 106	520 ± 72	601 ± 101	585 ± 94	590 ± 107	575 ± 95
CI (mg/kg bw/day)	-	13	73	371	-	17	84	410
F1B generation: FC over 12 weeks of dietary administration prior to mating, no treatment during gestation up to lactation Day 28 of the F2 generation								
FC (g/kg animal/week)	647 ± 168	638 ± 162	646 ± 177	657 ± 180	716 ± 135	696 ± 133	749 ± 139	737 ± 138
CI (mg/kg bw/day)	-	18	92	469	-	18	106	525
F1B generation: FC during individual weeks of dietary administration#								
Week 1	926	937	939	931	935	945	1030	933
Week 5	571	519	507	493	611	581	635	623
Week 6	722	654	696	678	766	725	805	779
Week 12	455	440	429	446	530	523	543	563
F2B generation: FC over 12 weeks of dietary administration prior to mating, no treatment during gestation up to lactation Day 28 of the F3 generation								
FC (g/kg animal/week)	697 ± 235	671 ± 228	685 ± 232	714 ± 252	742 ± 231	727 ± 231	751 ± 232	740 ± 211
CI (mg/kg bw/day)	-	19	98	510	-	20	107	528
F3C generation: FC over 8 weeks of dietary administration followed by 4 weeks of recovery								
FC (g/kg animal/week)	735 ± 248	740 ± 264	747 ± 256	721 ± 262	759 ± 187	745 ± 207	759 ± 184	776 ± 191
CI (mg/kg bw/day)	-	25	126	603	-	24	123	623
Mean achieved dose level over 3 generations								
CI (mg/kg bw/day)	-	17.8	92.2	462.2	-	19.2	106.0	502.0

FC: Food consumption; CI: Compound intake; #: no standard deviation reported

HAEMATOLOGY

Statistically significant findings were occasionally observed for all haematology parameters over all generations. Haemoglobin, haematocrit, red and white blood cell count, thrombocytes, reticulocytes, different types of granulocytes, coagulation time, eosinophils, lymphocytes and monocytes were currently found statistically significantly increased or decreased. The changes were either not dose-related, observed among animals of one sex only and/or not observed over all generations. Therefore, the observations were considered either not treatment-related or non-adverse and of no toxicological relevance.

CLINICAL CHEMISTRY

Biochemistry findings of statistical significance were occasionally observed among males and females of all generations and for all test item-treated and control groups. However, for none of the parameters investigated a dose-response relationship was observed, and the findings were not consistent over all examined generations.
Therefore, all clinical chemistry findings were considered either not treatment-related or non-adverse and of no toxicological relevance.

REPRODUCTIVE PARAMETERS

For F1A generation, the fertility index was reduced (-19% both) at 200 and 5000 ppm, and for the F1B dams the fertility index was reduced at ≥ 200 ppm (-17%, -17% and -33% at 200, 1000 and 5000 ppm, respectively). The observations at the low and intermediate dose level were not dose-related and therefore not attributed to treatment. The reduced fertility index at the highest dose level in the F1 generation was attributed to treatment, but in the absence of similar observations at the later generations, considered as non-adverse. For details on the fertility index please refer to the table below.

Table B.6.6.1/10-04: Fertility indices (%)

Generation	Dose group (ppm)			
	0	**200**	**1000**	**5000**
F1A*	83	67	83	67
% control	-	81	100	81
F1B	100	83	83	67
% control	-	83	83	67
F2A*	83	75	100	92
% control	-	90	120	111
F2B	92	100	83	100
% control	-	109	90	109
F3A*	96	96	92	88
% control	-	100	96	92
F3B*	83	96	96	83
% control	-	116	116	100
F3C	88	96	83	88
% control	-	109	94	100

* F1A, F2A, F3A, F3B refer to offspring

LITTER DATA

Number of pups delivered

There was no difference in the number of male and female live foetuses per litter between the 3 dose groups over all generations.

Viability index

The survival of pups between post-partum Days 0 and 4 was not affected by treatment. For all generations and dose levels, the viability index was comparable to those of control animals.

Table B.6.6.1/10-05: Viability indices (%)

Litter	Dose group (ppm)			
	0	**200**	**1000**	**5000**
F1A	100	100	100	100
F1B	100	100	100	100
F2A	100	95	96	88
F2B	100	99	98	100
F3A	100	100	97	98
F3B	100	99	100	100

Lactation index

When compared to control animals, a decrease in lactation index was observed in the 1000 and 5000 ppm groups of the F3B generation (-31% and -30%, respectively). The effect was caused by increased lethality in the animals of these groups during lactation Days 7 and 14. In addition, a reduction in lactation index was also noted for the F1A animals at 1000 ppm (-28%). The finding for the F1A intermediate dose group was not dose-related and therefore considered as incidental. The observations made for the F3B mid and high dose groups were not

observed for the F3A and F3C offspring and also not observed for the remaining generations, therefore the finding was considered as non-adverse.

Table B.6.6.1/10-06: Lactation indices (%)

Litter	Dose group (ppm)			
	0	**200**	**1000**	**5000**
F1A	82	87	59	100
% control	-	106	72	122
F1B	84	78	62	88
% control	-	93	74	105
F2A	100	95	96	88
% control	-	95	96	88
F2B	78	83	83	79
% control	-	106	106	101
F3A	98	93	87	82
% control	-	95	89	84
F3B	90	72	62	63
% control	-	80	69	70
F3C	93	86	82	84
% control	-	92	88	90

Post-natal survival index

In accordance with the lactation index, the post-natal survival index was decreased for F1A offspring at 1000 ppm (-28%) and for F3B offspring at 1000 and 5000 ppm (-32% and -31%, respectively). The finding for the F1A intermediate dose group was not dose-related and therefore not attributed to treatment. The observations made for the F3B mid and high dose groups were not observed for the F3A and F3C offspring and also not observed for the remaining generations. Hence, the observation was considered to be of no toxicological relevance.

Table B.6.6.1/10-07: Post-natal survival indices (%)

Litter	Dose group (ppm)			
	0	**200**	**1000**	**5000**
F1A	82	87	59	100
% control	-	106	72	122
F1B	84	78	65	88
% control	-	93	77	105
F2A	90	92	94	86
% control	-	102	104	96
F2B	84	82	81	91
% control	-	98	96	108
F3A	98	93	85	82
% control	-	95	87	84
F3B	90	72	61	62
% control	-	80	68	69
F3C	93	86	82	84
% control	-	92	88	90

Sex ratio

Sex ratio was not affected by treatment.

Body weights
Pup weights generally developed similar in the test item-treated groups and the control group. Growth rates were comparable to those of control animals for all three generations.

GROSS NECROPSY
In the animals of the F0/F0+ generation, there were no treatment-related macroscopic abnormalities observed.
In males of the F1B generation, thymus involution and clay-coloured liver was observed for all high dose group male animals. The observations were in line with histopathological abnormalities and attributed to treatment. In the male animals of the F1B generation, there was further an increased incidence of dilated renal pelvis/pyelectasia observed in the kidneys of the 1000 ppm animals. Similar observations were made for males of the F2B generation at 1000 and 5000 ppm (9/24 and 6/24 males vs. 2/24 males of the control group) and in 4/10 F3C males at 1000 ppm. The finding was less prominent in females and observed for only 1/24 females each at 1000 and 5000 ppm of the F2B generation and in each 1/10 females at 200, 1000 and 5000 ppm of the F3C generation. A toxic origin of the findings in the mid and high dose group animals of the F2B generation was not excluded, however, there was no clear dose-response relationship evidence, there were no consistent histopathological findings which may explain the findings.

Macroscopical abnormalities were further observed in the lungs of the F2B and F3C animals. Grey or yellowish-brown pinhead size spots of disseminated localisation were frequently observed in males and females, with increased incidences at the low and mid dose groups, but also findings in the control animals. Changes were also observed at histopathological examination, but without a dose-response relationship, the macroscopic findings were considered non-adverse.

In the F3C generation, there was an increased incidence of grits like, oedematous alterations in the pancreas. The finding was observed in males and females of control and test item-treated groups. In the absence of any dose-response relationship and as no findings in the pancreas were noted in the earlier generations, the macroscopic changes were considered not treatment-related.

Table B.6.6.1/10-08: Gross necropsy findings most frequently observed

Generation	Males				Females			
	0	200	1000	5000	0	200	1000	5000
Kidney: dilated renal pelvis/pyelectasis								
F0+	0/10	0/10	1/10	1/10	0/10	0/10	0/10	0/10
F1B	0/12	0/12	4/12	0/12	0/12	0/12	0/12	0/12
F2B	2/24	0/24	9/24	6/24	0/24	0/24	1/24	1/24
F3C	0/10	0/10	4/10	0/10	0/10	1/10	1/10	1/10
Lung: Pulmonary alterations: grey or yellowish brown pinhead size spots of disseminated localisation								
F0+	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
F1B	2/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
F2B	0/24	0/24	4/24	2/24	0/24	6/24	2-3/24#	3/24
F3C	3/10	4/10	6/10	2/10	2/10	6/10	1/10	0/10
Pancreas: Grits like, slightly edematous								
F0+	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
F1B	0/12	0/12	0/12	0/12	0/12	0/12	0/12	0/12
F2B	0/24	0/24	0/24	0/24	0/24	0/24	0/24	0/24
F3C	3/10	1/10	0/10	5/10	5/10	7/10	0/10	0/10
Thymus involution								
F0+	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
F1B	0/12	0/12	0/12	12/12	0/12	0/12	0/12	0/12
F2B	0/24	0/24	0/24	0/24	0/24	0/24	0/24	0/24
F3C	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Liver: clay-coloured (males only) or yellow with biliary ducts dilated to 3-4 fold size (females only)								
F0+	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

F1B	2/12	0/12	0/12	12/12	0/12	0/12	2/12	0/12
F2B	0/24	0/24	0/24	0/24	0/24	0/24	0/24	0/24
F3C	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

Organ weight

There were no statistically significant and dose-related changes in organ weights observed which were consistent over all generations. The most prominent alteration was a statistically significant increase in relative spleen weight, which was noted for test item-treated males of the F1B, F2B and F3C generation and in females of the F2B generation. However, there was no clear dose-response relationship evident and no abnormalities were observed at histopathological examination.

Also, for the adrenals, brain, heart, liver, kidneys, stomach and thymus, changes in relative organ weights were occasionally observed. However, the findings were noted for single generations only and without any dose-response relationship. Thus, a correlation to treatment with glyphosate was not evident.

Table B.6.6.1/10-09: Relative spleen weight changes (mean ± standard deviation)

Dose group	Males				Females			
(ppm)	0	200	1000	5000	0	200	1000	5000
Spleen weight#								
F0+	0.39 ± 0.04	0.39 ± 0.03	0.36 ± 0.06	0.39 ± 0.04	0.31 ± 0.02	0.30 ± 0.03	0.29 ± 0.04	0.33 ± 0.05
F0	0.40 ± 0.05	0.41 ± 0.05	0.44 ± 0.07	0.41 ± 0.07	0.32 ± 0.04	0.36 ± 0.06	0.35 ± 0.07	0.34 ± 0.03
F1B	0.42 ± 0.04	0.48 ± 0.12	0.47 ± 0.07	0.44 ± 0.04	0.36 ± 0.04	0.42 ± 0.07	0.43 ± 0.14	0.39 ± 0.05
F2B	0.34 ± 0.05	0.36 ± 0.05	0.40 ± 0.07	0.35 ± 0.04	0.31 ± 0.06	0.33 ± 0.05	0.34 ± 0.04	0.31 ± 0.04
F3C	0.34 ± 0.03	0.37 ± 0.05	0.38 ± 0.05	0.35 ± 0.05	0.28 ± 0.04	0.29 ± 0.04	0.32 ± 0.04	0.28 ± 0.03

#: Values not highlighted for statistical significance in the study report.

Table B.6.6.1/10-10: Relative organ weights (mean ± standard deviation) observed in F0+ generation males

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS
0	MEAN	1,76	0,37	0,19	0,83	0,31	3,41	0,79	0,041
	SD	0,07	0,02	0,05	0,07	0,02	0,20	0,06	0,006
200	MEAN	1,78	0,36	0,17	0,82	0,30	3,43	0,74	0,039
	SD	0,05	0,03	0,03	0,05	0,03	0,39	0,06	0,010
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	1,78	0,38	0,20	0,82	0,29	3,56	0,80	0,039
	SD	0,05	0,02	0,02	0,06	0,04	0,38	0,09	0,008
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
5000	MEAN	1,80	0,36	0,18	0,86	0,33	3,61	0,79	0,040
	SD	0,08	0,04	0,03	0,04	0,05	0,27	0,08	0,005
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT

Table B.6.6.1/10-12: Relative organ weights (mean ± standard deviation) observed in F0 generation males

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS	TESTIS	EPIDIDYMI
0	MEAN	2,02	0,59	0,18	1,13	0,40	6,70	1,42	0,029	2,00	0,70
	SD	0,04	0,07	0,04	0,07	0,05	1,40	0,13	0,003	0,21	0,06
200	MEAN	2,01	0,60	0,16	1,12	0,41	5,78	1,37	0,026	1,81	0,65
	SD	0,08	0,07	0,05	0,11	0,05	1,47	0,10	0,002	0,58	0,18
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	2,05	0,65	0,16	1,17	0,44	6,76	1,42	0,031	1,89	0,66
	SD	0,06	0,04	0,04	0,06	0,07	0,70	0,11	0,004	0,23	0,14
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
5000	MEAN	2,06	0,62	0,13	1,07	0,41	6,03	1,35	0,032	1,88	0,67
	SD	0,07	0,08	0,05	0,05	0,07	0,84	0,16	0,002	0,11	0,04
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT

Table B.6.6.1/10-13: Relative organ weights (mean ± standard deviation) observed in F0 generation females

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS
0	MEAN	1,85	0,47	0,18	0,98	0,32	4,14	0,90	0,045
	SD	0,10	0,03	0,04	0,05	0,04	0,41	0,06	0,008
200	MEAN	1,83	0,48	0,16	1,03	0,36	4,19	0,93	0,049
	SD	0,06	0,05	0,04	0,08	0,06	0,39	0,04	0,009
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	1,82	0,46	0,13	1,02	0,35	4,29	0,96	0,046
	SD	0,06	0,04	0,06	0,05	0,07	0,36	0,07	0,003
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
5000	MEAN	1,82	0,43	0,13	0,96	0,34	4,05	0,87	0,046
	SD	0,10	0,05	0,02	0,06	0,03	0,34	0,08	0,003
	T TEST	NS	NS	x	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT x : $P \leq 0,05$

Table B.6.6.1/10-14: Relative organ weights (mean ± standard deviation) observed in F1B generation females

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS
0	MEAN	1,868	0,462	0,170	0,950	0,360	4,375	0,926	0,043
	SD	0,094	0,064	0,023	0,083	0,044	0,427	0,084	0,005
200	MEAN	1,869	0,491	0,175	1,047	0,418	4,688	1,005	0,047
	SD	0,061	0,038	0,026	0,107	0,067	0,520	0,095	0,007
	T TEST	NS	NS	NS	x	x	NS	x	NS
1000	MEAN	1,879	0,498	0,179	1,015	0,431	4,581	0,921	0,046
	SD	0,116	0,056	0,063	0,103	0,137	0,577	0,126	0,005
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
5000	MEAN	1,850	0,503	0,171	1,010	0,398	4,552	0,943	0,048
	SD	0,074	0,044	0,027	0,079	0,048	0,502	0,093	0,004
	T TEST	NS	NS	NS	NS	NS	NS	NS	x

SYMBOLS
NS : NOT SIGNIFICANT x : P $\leq$ 0,05

Table B.6.6.1/10-15: Relative organ weights (mean ± standard deviation) observed in F2B generation males

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS	TESTIS	EPIDIDYMIS
0	MEAN	2,008	0,551	0,169	1,087	0,343	6,076	1,259	0,028	1,987	0,648
	SD	0,076	0,048	0,028	0,088	0,052	0,614	0,120	0,004	0,181	0,048
200	MEAN	2,063	0,526	0,181	1,071	0,357	6,047	1,190	0,025	1,960	0,642
	SD	0,197	0,043	0,051	0,149	0,045	0,731	0,083	0,004	0,202	0,067
	T TEST	NS	NS	NS	NS	NS	NS	x	x	NS	NS
1000	MEAN	2,022	0,610	0,164	1,100	0,396	6,102	1,275	0,029	2,049	0,740
	SD	0,062	0,053	0,042	0,122	0,073	0,847	0,148	0,004	0,148	0,050
	T TEST	NS	xxx	NS	NS	xx	NS	NS	NS	NS	xxx
5000	MEAN	2,023	0,538	0,170	1,076	0,352	5,902	1,210	0,029	1,912	0,623
	SD	0,094	0,042	0,044	0,149	0,036	0,616	0,130	0,004	0,222	0,075
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT x : P $\leq$ 0,05 xx : P $\leq$ 0,01 xxx : P $\leq$ 0,001

Table B.6.6.1/10-16: Relative organ weights (mean ± standard deviation) observed in F2B generation females

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS
0	MEAN	1,873	0,470	0,165	0,958	0,306	4,461	0,958	0,043
	SD	0,062	0,056	0,037	0,100	0,064	0,450	0,090	0,007
200	MEAN	1,862	0,476	0,171	0,989	0,328	4,443	0,959	0,041
	SD	0,084	0,037	0,041	0,078	0,050	0,509	0,081	0,005
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	1,885	0,499	0,150	0,938	0,343	4,336	0,953	0,044
	SD	0,083	0,049	0,041	0,061	0,044	0,485	0,082	0,006
	T TEST	NS	NS	NS	NS	x	NS	NS	NS
5000	MEAN	1,917	0,457	0,155	0,944	0,307	4,310	0,960	0,041
	SD	0,081	0,032	0,030	0,062	0,042	0,425	0,079	0,005
	T TEST	x	NS	NS	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT x : P $\prec$ 0,05

Table B.6.6.1/10-17: Relative organ weights (mean ± standard deviation) observed in F3C generation males

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS	TESTIS	EPIDIDYMIS
0	MEAN	1,927	0,480	0,235	0,997	0,340	5,632	1,205	0,032	1,869	0,602
	SD	0,053	0,047	0,043	0,104	0,032	0,415	0,078	0,006	0,230	0,058
200	MEAN	1,929	0,477	0,225	0,986	0,373	5,378	1,153	0,034	1,963	0,606
	SD	0,074	0,023	0,045	0,121	0,049	0,671	0,079	0,006	0,166	0,046
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	1,892	0,453	0,217	1,009	0,384	4,966	1,101	0,038	1,994	0,621
	SD	0,059	0,044	0,041	0,091	0,054	0,471	0,083	0,005	0,173	0,043
	T TEST	NS	NS	NS	NS	x	xx	xx	x	NS	NS
5000	MEAN	1,928	0,506	0,203	0,976	0,354	5,030	1,135	0,031	1,829	0,596
	SD	0,067	0,039	0,033	0,078	0,045	0,494	0,087	0,005	0,089	0,024
	T TEST	NS	NS	NS	NS	NS	xx	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT x : P $\prec$ 0,05 xx : P $\prec$ 0,01

Table B.6.6.1/10-18: Relative organ weights (mean ± standard deviation) observed in F3C generation females

DOSE MG/KG IN FOOD		BRAIN G	HEART	THYMUS	STOMACH	SPLEEN	LIVER	KIDNEYS	ADRENALS
0	MEAN	1,770	0,372	0,213	0,773	0,276	3,554	0,787	0,043
	SD	0,067	0,045	0,032	0,102	0,041	0,327	0,073	0,007
200	MEAN	1,790	0,358	0,209	0,785	0,285	3,419	0,732	0,041
	SD	0,081	0,046	0,023	0,069	0,042	0,270	0,080	0,005
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS
1000	MEAN	1,761	0,372	0,199	0,780	0,317	3,544	0,777	0,049
	SD	0,075	0,049	0,029	0,080	0,036	0,224	0,073	0,007
	T TEST	NS	NS	NS	NS	x	NS	NS	NS
5000	MEAN	1,804	0,363	0,188	0,811	0,278	3,375	0,774	0,044
	SD	0,035	0,029	0,038	0,060	0,028	0,293	0,055	0,004
	T TEST	NS	NS	NS	NS	NS	NS	NS	NS

SYMBOLS
NS : NOT SIGNIFICANT x : P = 0,05

Histopathology
At histopathological examinations, no dose-related findings of the same origin and appearance were observed for any organ, which was consistent over all generations. However, no information on individual animals were provided, and the exact number of animals affected per dose group for each organ among the generations examined was not always reported.

Following 3 months of treatment, findings in the liver such as swelling of parenchymal cell nuclei with chromatin set to the nuclear membrane, were observed in the animals of the F0+ generation. The findings were noted at ≥ 1000 ppm in males and at 5000 ppm in females. Further findings in the liver of F0+ animals comprised increased cell division and widened sinusoids, which were focally infiltrated with mononuclear cells. Such findings of the liver were not observed in F0 animals after weaning. Among the animals of the later generations, the most frequent observation in the liver was parenchymal/peripheral vacuolisation. Further abnormalities comprised abscesses, hemosiderosis or perilobular cell infiltration. Any findings observed occurred without a clear dose-response relationship and there was no consistency over all generations.

In the F1B generation, thymus involution/ ack of medullary substance was reported for males and females of the high dose group. The observation in males was consistent with macroscopical findings. Further abnormalities in the thymus that were frequently observed, comprised cysts containing colloids and inflammatory cells or tiny bleedings in the cortical and/or medullary substances. The latter findings were noted among all generations but without any dose response relationship and were therefore considered not treatment-related.

All test item-treated F1B animals further showed a widened gastric mucosa and fundus mucosa in the stomach. In the low (200 ppm) and mid dose group (1000 ppm), increased incidences of small bleedings and/or superficial erosions in the glandular stomach of the antrum was observed.

Similar findings in the stomach were also observed in the F0 generation (in males at 200 and 5000 ppm) and in the F2B generation (in males and females at ≥ 1000 ppm). In addition, eosinophyl ulcer was noted in the antrum in males and females of the F0+, F2B and F3C generation, but were noted in treated as well as control animals.
In the pancreas of all generations, fatty degeneration of the exocrine glandular tissue was frequently observed. The findings were noted in control and test item-treated animals of the F0, F1B and F2B generations, with an extent of higher degree in the glyphosate-treated animals. In a few animals, the observation was accompanied by nodular islet cell hyperplasia. In the F3C generation, partially vacuolised exocrine substance was noted in males at 0 and 200 ppm and in females at 1000 and 5000 ppm.

For any finding, a relation to treatment with glyphosate could not be excluded. However, there was no dose-response relationship and none of the effects was consistently observed over all generations. Findings in the

thymus and degeneration of the pancreas were reported to be not reversible, however, incidences of histopathological observations in these organs were not reported for F3C animals.

The abnormalities observed for lung at gross macroscopical examination in the F2B and F3C generation were identified as a mixture of histopathological findings comprising perivascular and peribronchial lymphoid hyperplasia, alveolar macrophages, increased macrophagic activity, proliferation of the respiratory epithelium, giant cell diapedesis-type bleeding and bronchial epithelium proliferation. However, in the absence of any dose response relationship a relation to treatment with glyphosate remained unclear.

In the kidneys of the 1000 and 5000 ppm dose group F1B, F2B and F3C animals, for which dilatation of renal pelvis/pyelectasia was observed at macroscopical examination, there were no histopathological abnormalities observed explaining these alterations. Sclerotical inclusions or foci in the corticomedullar boundary were commonly observed in the majority of males or females in the F1B and F2B generation, but no findings were noted in the F3B generation. In addition, cortical retractions caused by nephron necrosis was observed in a few male animals of the F2B generation at 200 and 1000 ppm, but there were no abnormalities which may explain the macroscopical findings noted in the high dose group animals of this generation.

For details on histopathological abnormalities that were most frequently observed, please refer to the table below.

Table B.6.6.1/10-19: Incidences of selected histopathological findings *

Generation	Histopathological finding
Liver	
F0+	Swollen, light colored parenchymal cell nuclei with chromatin set to the nuclear membranes in 3/3 males and 3/3 females at 1000 ppm and in 3/3 females at 5000 ppm Findings accompanied with karyolysis, sinusoidal widening and mononuclear infiltration
F0	Cells with significant focal parenchymal vacuolisation in 20% of the animals of all groups Perilobular vacuolisation of 60% of the tissue in females of all groups, no dose-relation
F1B	Peripheral vacuolisation and perivascular infiltration in all animals Abscess in 1/5 females at 200 ppm Hemosiderosis in 1/5 females at 1000 ppm
F2B	Vacuolisation of the peripheric zone in 1/5 males at 1000 and 2/5 males at 5000 ppm and in females at 200 and 1000 ppm Mild round cell infiltration in the peripheric zone with focal appearance of round cells in the sinusoids in 30% of the females of the 1000 and 5000 ppm groups
F3C	Centrilobular infiltration and focal necrosis in 2/5 and 2/5 males at 0 and 200 ppm Perilobular infiltration in 2/5 and 3/5 females at 200 and 1000 ppm Vacuolisation in 1/5 males and 2-3/5 females at 5000 ppm Carbohydrates diminished and disappeared in males and females at 5000 ppm
Thymus	
F0+	Cysts containing colloid and inflammatory cells in 1/3 and 1/3 males at 200 and 1000 ppm and in 1/3 and 1/3 females at 200 and 5000 ppm Tiny bleedings of the corticomedullar boundaryin 1/3 males at 1000 ppm
F0	50% tissue involution in 1/5 control males Colloid-containing cysts, surrounded by epithelial cells in 2/5 and 3/5 males at 200 and 5000 ppm Dose unrelated small bleedings in the cortical and/or medullary substances (in 60% all groups)
F1B	Meduallary substance almost missing in 1/5 males and 1/5 females at 5000 ppm Involution in high dose group males Epithelial cysts, 20%, dose-unrelated incidence in females
F2B	Cysts filled with colloid and bound with epithelial cells in a few animals/sex/group Fatty involution in 60% of control males

Generation	Histopathological finding
F3C	Small bleedings in the corticomedullar boundary, 40%, dose unrelated incidences in the male groups Tiny cysts filled with colloid in 50% of the females
Stomach	
F0+	Eosinophil ulcer in 2/2 males at 0 ppm, in 1/3 males and 1/3 females at 5000 ppm
F0	Oedematous widening of the fundus mucosa in 1/5 males at 200 ppm and in 3/6 males at 5000 ppm
F1B	Loosened, widened submucosa in the antrum fundus and focal peritubular interstitial infiltration in the glandular part of the antrum in males and females Local bleeding in the surface of the glandular part of the antrum in 1/5 males at 200 ppm and 1/5 females at 1000 ppm Tiny superficial erosions in the fundus in 2/5 males at 200 ppm
F2B	Widerend submucosa in 1/5 and 3/5 males and females at 1000 and 5000 ppm fundus accompanied by mild focal round cell infiltration Eosinophil ulcer in the antrum in 1/5 males and 2/5 females at 200 ppm and in 1/5 females at 5000 ppm
F3C	Vacuolisation in acid cells in the glandular antrum in males at 1000 ppm and females at 200 - 5000 ppm Eosinophil ulcer in the antrum in 1-2 males and females per group each at 200 and 1000 ppm
Pancreas	
F0+	Nodular hyperplasia, slight mononuclear interstitial infiltration in the LH insula in 1/5 males each at 1000 and 5000 ppm Focal necrosis in the exocrine substance in 1 male animal Fatty degeneration, dose un-related but with higher incidence in treated groups
F0	Fatty degeneration in 70% of the exocrine glandular tissue and focal insular hyperplasia in 1/5 females at 200 ppm
F1B	Focal insular hyperplasia in 1/5 males at 200 ppm
F2B	Focal necrosis in the exocrine substance in 2/5 males at 0 ppm, in 1/5 females at 200 ppm and in 1/5 males at 1000 ppm Fatty degeneration in > 30% of the exocrine substance in 1/5 animals of each dose group Perivascular mononuclear cell infiltration was occasionally observed
F3C	Partially vacuolised exocrine substance in 1/5 and 2/5 males at 0 and 200 ppm and for 1/5 and 2/5 females at 1000 and 5000 ppm
Lung	
F0+	Perivascular, -peribronchial lymphoid hyperplasia in 1/6 males and 1/6 females at 0 ppm Alveolar macrophage groups 1 male in each group, 1 female each at 200 and 1000 ppm Edematous cell-rich alveolar septa widening into 30 - 80% of the surrounding tissues in each 1 male and female at 200 ppm and each 1 female at 1000 and 5000 ppm Congestion in 1/3 females at 5000 ppm Diapedesis-type bleedings in 1/3 males and 1/3 females at 200 ppm
F0	Perivascular, -peribronchial lymphoid hyperplasia in 5/5 males and 4/5 females at 0 ppm Cell-rich alveolar septa widening into 40-50% of the surrounding tissues in females, 3/5, 1/5 and 1/5 at 200, 1000 and 5000 ppm
F1B	Perivascular, -peribronchial lymphoid hyperplasia (30%, dose unrelated incidence in all groups Alveolar macrophage groups in 2 males and 2 females each Edematous cell-rich alveolar septa widening into 50-75% of the surrounding tissues and compensatory emphysema (30%, dose unrelated, observed in all groups) Congestion in 1/5 males each at 200 and 1000 ppm Focal interstitial pneumonia in 2/5 females at 5000 ppm

Generation	Histopathological finding
F2B	Perivascular, -peribronchial lymphoid hyperplasia in 10% of the animals Alveolar macrophage groups in 1/4 and 2/3 part of males and females Increased macrophagic activity in 1-2 females/group Proliferation of the respiratory epithelium in 2/5 females at 200 ppm
F3C	Interstitial pneumonia, 40% and 20%, dose unrelated incidence in the male and female groups Peribronchial, perivascular lymphocytic hyperplasia and increased macrophagic activity in 2/3 of the animals Giant cells diapedesis-type bleeding in 1 animal per group Bronchial epithelium proliferation in 1/5 males at 5000 ppm
Kidney	
F0+	Focal nephron degeneration, 30% dose unrelated incidence in male groups Vacuolic degeneration in 3 males and 3 females Sclerotic inclusions in the corticomedulary boundary in 1/5 females at 0 ppm and 1/5 females at 200 ppm
F0	No pathological alterations
F1B	Sclerotic inclusions in the corticomedullar boundary, 30%, dose unrelated incidence in all female groups
F2B	Cortical retractions caused by nephron necrosis in each 2/5 and 2/5 males at 200 and 1000 ppm Sclerotic foci in the corticomedullar boundary in 2/3 of all males
F3C	No pathological alterations
Spleen	
F0+	Not findings reported
F0	Hemosiderin accumulation and extramedullar hemopeisis in inflammatory infiltration in the red pulp, occasional, incidence of 0-30% in all groups
F1B	Hemosiderin in the red pulp in all animals Extramedullar hemopoiesis, 25%, dose unrelated in all male groups Inflammatory infiltration, 30%, dose unrelated in all female groups
F2B	Hemosiderin in the red pulp increased in all animals, especially in females
F3C	Widened white pulp in 2/5 males at 0 ppm, in 1/5 males and 2/5 females at 200 ppm and in 2/5 males and 2/5 females at 5000 ppm Inflammatory infiltraton in the red pulp and occasional hemosiderin accumulation, 30%, dose unrelated incidence

* No further details on the number of animals affected per group and on severity of any abnormality observed.

CONCLUSIONS (STUDY AUTHOR)
Oral administration of glyphosate to rats by dietary administration at a top dose level of 5000 ppm over three successive generations was not associated with any adverse effects on fertility, general reproductive performance or litter data. In addition, no treatment-related effects of systemic toxicity were noted up the highest dose level tested.

Therefore, the highest dose level of 5000 ppm (corresponding to mean achieved doses of 462.2 mg/kg bw/day in males and 502.0 mg/kg bw/day in females) was considered as 'No Observed Adverse Effect Level' (NOAEL).

Assessment and conclusion by applicant:
In the present study, glyphosate technical was administered to male and female Wistar rats at doses of 200, 1000 and 5000 ppm over three consecutive generations. The study is a pre-guideline study and was conducted before GLP was compulsory. The study has several reporting deficiencies: the design of the study is not accurately described, Moreover, no identification number was assigned to the animals making it difficult to monitor the fate of the treated animals and correlate the offspring with their parents.

Only a limited set of parameters on systemic as well as reproductive toxicity have been examined.

No details on the test material (batch and purity) were provided in the study report and no analytical determinations on stability and homogeneity of the test material in the diet was conducted. No justification for the selection of dose levels was given and when compared to other repeated dose toxicity/reproductive and developmental toxicity studies, the top dose level of the present study (5000 ppm, corresponding to approx. 500 mg/kg bw/day) was rather low.

No data on individual animals or summarised data were provided on clinical signs of toxicity and mortality of adult animals. Statistical analysis was only conducted for some of the parameters investigated and not always indicated in result tables. In addition, the starting body weight of F0+ animals was statistically significantly higher for glyphosate-treated animals than for control animals. When compared to currently valid guidelines, a limited set of parameters for haematology and clinical chemistry was investigated. The oestrus cycle of the animals was not monitored and no information on time to mating or confirmation of pregnancy was reported. No absolute but only relative organ weights were reported. In addition, there were no historical control data included in the study report. Due to the large number of deviations and reporting deficiencies, the study was considered insufficient for assessment.

Assessment and conclusion by RMS:

Glyphosate was given in the diet at dose levels of 0, 200, 1000 and 5000 ppm to Wistar rats over three generations. The control group received plain diet only. In the F1 and F2 generations there were two mating. In the F3 generation three litters were produced. In the F3c generation, 10 animals per sex and dose group were selected by randomisation and examined following an 8-week dosing period and a subsequent 4-week recovery phase. A limited number of rats (3 to 5 per dose group and generation) was subjected to histopathology.

Treatment was associated with following findings:

- reduced fertility index observed at 5000 ppm (F1A: 67% compared to 83% in control; F1B:67% compared to 100% in control)
- reduced lactation index (at 1000 ppm: F3B: 31%; at 5000 ppm: F3B 30%)
- macroscopical changes in thymus (involution, F1B males at 5000 ppm
- macroscopical changes in the liver (clay-coloured, F1B males at 5000 ppm)
- histopathological changes in thymus (involution, F1B males at 5000 ppm)
- histopathological changes in liver (peripheral vacuolisation and perivascular infiltration in all FIB animals)
- histopathological changes in stomach (at 200 ppm: widened gastric mucosa and fundus mucosa (F1B males and females), increased incidences of small bleedings in the glandular stomach of the antrum (F1B males and females); at 1000 ppm: widened gastric mucosa and fundus mucosa (F1B and F2B males and females), increased incidences of small bleedings in the glandular stomach of the antrum (F1B and F2B males and females); at 5000 ppm: widened gastric mucosa and fundus mucosa (F1B and F2B males and females))
- histopathological changes in pancreas (fatty degeneration observed in F0, F1B and F2B generation animals at 200, 1000, 5000 ppm)

The study is not acceptable (reporting deficiencies and major deviations from OECD TG 416).

Following deviations from the OECD TG 416 (2001) were observed:
(i) purity not specified
(ii) lot/batch number not specified
(iii) no analytical determinations on stability and homogeneity of the test material
(iv) mating period was 6 consecutive days (the guideline recommends a 2-week mating period)
(v) during mating, males changed daily so that each female cohabited with different males (the guideline recommends for each mating each female should be placed with a single male from the same dose level (1:1 mate) until copulation occurs 2 weeks have elapsed).
(vi) age of F0 animals at initiating of dosing was 28 days (the guideline recommends the animals to be 5 to 9 weeks old at the start of dosing)
(vii) few animals were used. Six F0 animals/sex/dose group were used for production of the F1 generations, F1A and F1B generations. Twelve animals/sex/dose groups of the F1B generation were mated to get F2 generations (the guideline recommends each test and control group should contain a sufficient number of

animals to yield preferable not less than 20 pregnant females at or near parturition)
(viii) oestrous cycle monitoring not performed
(ix) pre-coital interval not recorded
(x) no sperm analyses
(xi) a quantitative evaluation of primordial follicles not conducted
(xii) sexual maturation not investigated in offsprings
(xiii) uterus, ovaries, seminal vesicles with coagulating glands, pituitary and thyroid not weighed
(xiv) vagina, uterus with cervix, ovaries, testis, epididymidis, prostate, seminal vesicles, coagulating gland not included in the histopathological examination for adults

ED related parameters* not investigated in study:
*parameters for OECD TG 416 study are listed in Table 14 in the EFSA guidance document for the identification of endocrine disruptors in biocides and pesticides (EFSA Journal 2018; 16(6):5311)

EATS-mediated parameters not investigated:

- thyroid weight
- age at balanopreputial separation
- age at vaginal opening
- anogenital distance
- oestrous cyclicity
- sperm morphology
- sperm motility
- sperm numbers
- cervix histopathology
- coagulating gland histopathology
- coagulating gland weight
- ovary weight
- prostate histopathology
- prostate weight
- seminal vesicles weight
- uterus weight
- uterus histopathology
- vagina histopathology
- vaginal smears

Sensitive to but not diagnostic of EATS:

- gestation length
- pituitary weight
- presence of anomalies
- time to mating
- litter size
- number of implantations (corpora lutea)
- post-implantation loss
- pre-implantation loss
- pup development

B.6.6.1/14

Data point	CA 5.6.1/014
Report author	[redacted]
Report year	1981
Report title	A three generation reproduction study in rats with glyphosate
Report No.	77-2063
Document No.	M-644052-01-1
Guidelines followed in study	None (pre-guideline)

Deviations from current test guideline	Not applicable Comments by RMS: The study was checked for compliance with OECD TG 416 and following deviations were observed: • parental animals were dosed 63 days (males) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period) • animals were mated in a sex ratio of 1 male:2 females to produce the F1 litters (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating)) • dose levels tested much too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering) • no information on pre-mating dosing period • oestrous cycle monitoring not performed • pre-coital interval not recorded • no sperm analyses • a quantitative evaluation of primordial follicles not conducted • physical and sexual maturation not investigated in offsprings • thymus of pups not weighed
Previous evaluation	Not accepted in RAR (2015)
GLP/Officially recognised testing facilities	No (pre-GLP)
Acceptability/Reliability	No (See RMS´ assessment below)

MATERIALS AND METHODS

Test material:	Glyphosate
Lot/Batch:	XHJ-64 Stability of test compound: Not reported
Purity/Radiochemical purity:	Purity: considered 100% active ingredient for dosing preparations
Vehicle:	Plain diet
Animals:	Species: Rat Strain: CD® (Sprague-Dawley derived) Age at start of treatment: 43 days Sex: Males and females Mean body weight at initiation of dosing: Males: 139.9 - 144.3 g; females: 118.0 - 119.2 g Acclimation period: 7 days Diet/Food: Standard laboratory diet (Purina Lab Chow® 5001), *ad libitum* Water: Automated watering system (Elizabethtown Water Company), *ad libitum* Housing: Individually (except during mating and lactation), in elevated stainless steel wire mesh cages; nesting material: Litter Kleen® hardwood shavings added to cages on Day 19 of gestation and changed when wet or soiled through Day 14 of lactation Environmental conditions: Temperature: not reported Humidity: not reported Air changes: not reported 12 hours light/dark cycle

STUDY DESIGN

Animal assignment and treatment
In a three generation reproduction study groups of 12 male and 24 female CD rats received beginning 63 days prior to mating of the F0 generation daily dietary doses of 0, 3, 10 and 30 mg glyphosate/kg bw in diet. Diet samples were taken at four week intervals for analysis of achieved test substance concentrations.

Mating
One male and two females of equivalent dose levels were caged together nightly until a sign of mating (sperm and/or copulation plug in the vagina) was observed or until 15 days had elapsed with no evidence of mating. The day on which evidence of mating was observed was defined as Day 0 of gestation.

In this study, the first litters (F1a, F2a and F3a) from each mating were raised to weaning and discarded. Rats produced by the second matings (F1b and F2b) were selected to become parents of succeeding generations or to be subjected to complete gross necropsy (F3b).

Table B.6.6.1/11-01: Study design

Group	Dose level (mg/kg bw/day)	No. of adults initially assigned to mate F0, F1, F2		No. of matings per generation F0, F1, F2	Gross post mortem examination	Histopathology of F0, F1 and F2 parents, F3b weanlings	
		Males	Females			Male	Female
1 Control (plain diet)	0	12	24	2	All	10	10
2	3	12	24	2		none	none
3	10	12	24	2		none	none
4	30	12	24	2		10	10

Diet preparation and analyses
Diets were prepared weekly during the study and were adjusted on the basis of body weight and food consumption.

Clinical observations
A check for clinical signs of toxicity and mortality was made twice daily. A detailed physical examination was performed on adult generations at weekly intervals throughout the study.

Body weight
Body weights of all animals were determined weekly during growth and rest periods of all generations. Pregnant females were weighed on Days 0, 6, 15 and 20 of gestation and lactating females were weighed on Days 0, 4, 14 and 21 of lactation.

Food consumption and compound intake
Food consumption was recorded weekly during growth and rest periods of all generations. Test substance intake was calculated from individual body weight and food consumption data and reported as a group mean value for weekly intervals during the growth and rest periods of all generations.

Reproduction parameters
The day on which evidence of mating was observed was designated as Day 0 of gestation; the day of delivery was designated as Day 0 of lactation.

Mating indices, pregnancy rates, length of gestation and male fertility indices were recorded.

Litter data
Pups of all generations were examined daily for general appearance and mortality. On Days 0, 4, 14, and 21 they were counted to record the number of live and dead pups. Body weights were determined on Days 0, 4, 14, and 21 as a litter and on Day 21 individually.

Total number of live and dead pups, and the number of males and females per litter were determined on Day 0 of lactation. The sex ratio was calculated for each group on Days 0 and 21 of lactation. Viability indices, were determined for each litter on Lactation Days 0, 4 and 21.

Sacrifice and pathology
Animals of all generations that died, were found dead or were killed moribund during the study period were necropsied as soon as possible. All adult males and females were sacrificed after pup selection of the last Fb litter (F0, F1) and after last F3b litter weaned (F2) by lethal exposure to ether. Pups that were found dead or stillborn pups were weighed and given a gross post mortem examination including internal sex determination, presence of milk in stomach. F1a, F2a, F3a and F3b animals were sacrificed at weaning, given a gross post mortem examination and abnormal tissues were saved. F1b and F2b animals which were not selected as future parents were sacrificed after ensuing selection of parental animals, given a gross post mortem examination and abnormal tissues were saved.

The following organs and tissues were preserved from all parents (F0, F1, F2) and from 10/sex/group of the F3b weanlings: adrenals, aorta, bone and bone marrow (sternal), brain, colon, duodenum, eyes with optic nerve and Harderian gland, gonads (ovaries and testes), heart, ileum, kidney (2), liver (2 sections), lung with main stem bronchi, lymph nodes (mesenteric), mammary gland (right inguinal), pancreas, pituitary, salivary gland, skeletal muscle (biceps femoris with right sciatic nerve), skin, spinal cord, spleen, stomach, thyroid/parathyroid, urinary bladder, uterus/prostate, gross lesions, tissue masses, thymus.

Microscopic examination of histological sections of these tissues were done for 10 male and 10 female animals from control and high-dose groups of F0, F1 and F2 parents and of F3b offspring (Aorta-thoracic region saved but not evaluated microscopically. Lumbar spinal cord was encased in vertebral column and was not evaluated microscopically).

The following organs were weighed from all parents sacrificed after weaning of the second litters and from eighty F3b weanlings (10 males and 10 females per group): adrenals, gonads, kidneys, brain, spleen, liver, heart and pituitary.

All pups of the second litter of the F2 parents (F3b) were necropsied at weaning and specified tissues were preserved for selected animals in each group.

Statistics
Body weights, body weight gain, maternal body weights, food consumption, number of offspring, offspring body weights, terminal body weights and organ weight data (absolute and relative), offspring survival, litter survival, pup viability index at birth, mating indices, pregnancy rates and male fertility indices data were compared to the control. Statistically significant differences were evaluated using several methods including Dunnett's test, ANOVA, Barlett's test, Kruskal-Wallis test and Fisher Exact Test.

RESULTS AND DISCUSSION

ANALYSIS OF DOSE FORMULATIONS
Not reported.

MEAN WEEKLY TEST SUBSTANCE INTAKE
Mean weekly test substance intake values ranged from 2.8 to 3.3 mg/kg bw/day for the low-dose group, from 9.5 to 11.2 mg/kg bw/day for the mid-dose group and from 27.7 to 33.1 mg/kg bw/day for the high dose group for all generations including both genders.

	Males			Females		
Group[a]	II	III	IV	II	III	IV
F_0 Growth	2.9	9.7	29.2	3.0	10.1	29.8
F_0 Rest	3.0	10.0	30.1	2.8	9.6	29.0
F_1 Growth	3.3	11.2	33.1	3.3	11.2	33.1
F_1 Rest	2.9	9.7	28.4	2.8	9.5	27.7
F_2 Growth	3.1	9.9	31.1	2.8	9.2	27.9
F_2 Rest	2.9	9.9	30.1	3.0	9.5	29.5

[a]Batch ratio for weekly diet preparations during the growth and rest periods were adjusted to achieve dose levels of 3, 10 and 30 mg/kg (both sexes).

MORTALITY

F0 adults (2 dead females in mid-dose group)

In the F0 generation, no unscheduled mortality occurred in the control, low- or high-dose groups. One female of the mid-dose group died during on Lactation Day 20 of first litter having 13 live pups at time of death. A second female of the mid-dose group died on Lactation Day 7 of second litter; this female delivered eight pups - seven live and one dead - and all pups were dead at time of death. No mid-dose F0 male died.

F1 adults (1 dead female in mid-dose group, 1 dead female in high-dose group)

In the F1 generation, no unscheduled mortality occurred in the control or low-dose groups. In the mid dose group one female was killed in a moribund condition during the post-mating period for the second litter. This female had mated during the first mating but did not deliver a litter; during the second mating this female had not mated. No other mortality occurred in the mid-dose group. In the high dose group one female died due to an accident (animal was caught in the feeder jar). A second high-dose female died on Day 21 of gestation for the second litter; the uterus of this female contained 15 term foetuses. No other mortality occurred in the high-dose group.

F2 adults (1 dead female in low-dose group, 1 dead male in mid-dose group)

In the F2 generation, no unscheduled mortality occurred in the control or high-dose groups. In the low-dose group one female died during the F3a lactation period. This female delivered a litter containing only dead pups (13 pups) and died the day after parturition. No other mortality occurred in the low-dose group. In the mid-dose group one male was killed in a moribund condition during the period between mating of the first and second litters. This male had mated and impregnated both females during the first mating period. No other mortality occurred in the mid dose group.

CLINICAL OBSERVATIONS

Clinical observation data were similar between the control and treated groups for each generation interval throughout the study. No adverse treatment effects were indicated.

BODY WEIGHT

Mean body weight data during the growth and rest periods were comparable between the control and treated groups for each generation, throughout the study. Likewise, mean weight gain during the growth periods were comparable between these same groups for both sexes throughout all generations. No treatment effect on body weight data during the growth and rest periods was evident.

Maternal body weights

Mean body weight data during the gestation and lactation intervals and mean weight change during these same periods were comparable between the control and treated group for each pregnancy interval from each generation throughout the study. No treatment effect was indicated in gestation - lactation body weight data throughout the study.

Offspring body weights

Mean pup body weight data during each litter interval for each generation were comparable between the control and treated groups. No adverse effects of treatment on pup weight data was evident.

Adult animals (F0, F1 and F2)
Mean terminal body weight data were comparable between the control and treated groups for both males and females throughout the study.

FOOD CONSUMPTION AND TEST COMPOUND INTAKE
Mean food consumption data were considered comparable between the control and treated groups (both sexes) during the growth and rest periods for each generation, throughout the study. No adverse effect of treatment on food consumption was evident throughout the study.

REPRODUCTIVE PARAMETERS
Male and female mating indices and male fertility indices during both mating intervals of the F0 generation were considered comparable between the control and treated groups. During the second mating interval of the F0, pregnancy rates were lower than control in each of the treated groups; however, no indication of a dose-relationship was evident as the lowest pregnancy rate was seen in the mid-dose group. This reduction in pregnancy rate for the mid-dose group was not statistically significant. In the absence of a dose-response relationship the reduction in pregnancy rate during this mating interval (F1b) in the treated groups was not considered treatment-related.

In the F1 generation, mating indices (males and females) for both litter intervals were comparable between the control and treated groups. It is note-worthy that for both mating intervals of this generation, mating indices for control and some treated groups were lower than normally encountered in multi-generation studies. The reason for the poorer mating performance in this generation was unclear but no treatment effect was indicated since mating indices were lowest in the control group. Pregnancy and male fertility indices for the first mating interval of the F1 were comparable between the control and treated groups. During the second litter interval, pregnancy rates were lower than those seen for the first interval in control and treated groups. The lowest pregnancy rate was seen in the high-dose group; however, this difference from the control value was not statistically significant. Pregnancy rates for the low- and mid-dose groups, during the second mating interval, were considered comparable to control. Male fertility indices for this same mating interval were considered comparable between the control and treated groups.

In the F2 generation mating indices for the treated groups were lower than control for each mating interval. During the first mating interval of the F2 generation, the female mating indices were lower than control in each of the treated groups; however, only in the high-dose group was this difference from control statistically significant. The female mating index for the control group at this interval was 100% which is higher than normally encountered. The female mating indices observed for the control group in this study have shown considerable variability ranging from 70.9 to 100% The poor mating performance for the treated groups during the first mating interval is attributed to two males in each treatment group that did not mate either female in their mating unit (each mating unit was comprised of one male and two females).

During the second mating interval of the F2 generation, male mating performance improved in the mid- and high-dose groups as both mid-dose males and one of two high-dose males that did not mate during the first mating interval, mated and impregnated at least one female. Male mating indices for the low-dose group remained unchanged as the same two males that did not mate during the first interval, failed to mate during the second interval. Pregnancy and fertility indices for the treated groups were comparable to control for both litter intervals of the F2 generation.

Table B.6.6.1/11-02: Mortality, mating, pregnancy and fertility rates

Group mg/kg/day	Total Number Exposed Females	Total Number Exposed Males	Mortality[a] Females No.Dead	Mortality[a] Females %	Mortality[a] Males No.Dead	Mortality[a] Males %	Mating Females Mated[b]/Total No.	Mating Females Mated[b]/Total %	Mating Males Mated[c]/Total No.	Mating Males Mated[c]/Total %	Pregnancy Females Pregnant/No. Mated No.	Pregnancy Females Pregnant/No. Mated %	Fertility Males Impregnating[d]/No. Mated No.	Fertility Males Impregnating[d]/No. Mated %
F_0 Mating (for F_{1a} generation)														
I 0	24	12	0	0.0	0	0.0	20/24	83.3	11/12	91.7	19/20	95.0	11/11	100.0
II 3	24	12	0	0.0	0	0.0	22/24	91.7	12/12	100.0	21/22	95.5	12/12	100.0
III 10	24	12	1	4.2	0	0.0	19/24	79.2	10/12	83.3	16/19	84.2	9/10	90.0
IV 30	24	12	0	0.0	0	0.0	21/24	87.5	11/12	91.7	19/21	90.5	11/11	100.0
F_0 Mating (for F_{1b} generation)														
I 0	24	12	0	0.0	0	0.0	20/24	83.3	11/12	91.7	19/20	95.0	11/11	100.0
II 3	24	12	0	0.0	0	0.0	23/24	95.8	12/12	100.0	19/23	82.6	12/12	100.0
III 10	23	12	1	4.3	0	0.0	17/23	73.9	11/12	91.7	12/17	70.6	10/11	90.9
IV 30	24	12	0	0.0	0	0.0	22/24	91.7	11/12	91.7	18/22	81.8	10/11	90.9

No statistically significant differences from control.
[a]Includes growth, gestation, lactation and rest periods.
[b]Number of females showing evidence of mating plug and/or sperm and/or pregnancy.
[c]Number of males for which mating was confirmed in at least one female.
[d]Number of males mated with at least one female for which parturition was evident.

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Group mg/kg/day	Total Number Exposed Females	Total Number Exposed Males	Mortality[a] Females No.Dead	Mortality[a] Females %	Mortality[a] Males No.Dead	Mortality[a] Males %	Mating Females Mated[b]/Total No.	Mating Females Mated[b]/Total %	Mating Males Mated[c]/Total No.	Mating Males Mated[c]/Total %	Pregnancy Females Pregnant/No. Mated No.	Pregnancy Females Pregnant/No. Mated %	Fertility Males Impregnating[d]/No. Mated No.	Fertility Males Impregnating[d]/No. Mated %
F_1 Mating (for F_{2a} generation)														
I 0	24	12	0	0.0	0	0.0	18/24	75.0	10/12	83.3	18/18	100.0	10/10	100.0
II 3	24	12	0	0.0	0	0.0	23/24	95.8	12/12	100.0	20/23	87.0	11/12	91.7
III 10	24	12	0	0.0	0	0.0	18/24	75.0	9/12	75.0	17/18	94.4	9/9	100.0
IV 30	23	12	1	4.2	0	0.0	19/23	82.6	11/12	91.7	18/19	94.7	10/11	90.9
F_1 Mating (for F_{2b} generation)														
I 0	24	12	0	0.0	0	0.0	17/24	70.8	9/12	75.0	15/17	88.2	9/9	100.0
II 3	24	12	0	0.0	0	0.0	19/24	79.2	10/12	83.3	15/19	78.9	9/10	90.0
III 10	24	12	0	0.0	0	0.0	17/24	70.8	10/12	83.3	14/17	82.4	10/10	100.0
IV 30	23	12	1	4.3	0	0.0	19/23	82.6	12/12	100.0	14/19	73.7	10/12	83.3

No statistically significant differences from control
a Includes growth, gestation, lactation and rest periods
b Number of females showing evidence of mating: plug and/or sperm and/or pregnancy
c Number of males for which mating was confirmed in at least one female
d Number of males mated with at least one female for which parturition was evident

Group /kg/day	Total Number Exposed Females	Total Number Exposed Males	Mortality[a] Females No. Dead	Mortality[a] Females %	Mortality[a] Males No. Dead	Mortality[a] Males %	Mating Females Mated[b]/Total No.	Mating Females Mated[b]/Total %	Mating Males Mated[c]/Total No.	Mating Males Mated[c]/Total %	Pregnancy Females Pregnant/No. Mated No.	Pregnancy Females Pregnant/No. Mated %	Fertility Males Impregnating[d]/No. Mated No.	Fertility Males Impregnating[d]/No. Mated %
						F_2 Mating (for F_{3a} generation)								
I 0	24	12	0	0.0	0	0.0	24/24	100.0	12/12	100.0	23/24	95.8	12/12	100.0
II 3	24	12	1	4.2	0	0.0	20/24	83.3	10/12	83.3	20/20	100.0	10/10	100.0
III 10	24	12	0	0.0	0	0.0	20/24	83.3	10/12	83.3	16/20	80.0	8/10	80.0
IV 30	24	12	0	0.0	0	0.0	18/24	75.0*	10/12	83.3	17/18	94.4	10/10	100.0
						F_2 Mating (for F_{3b} generation)								
I 0	24	12	0	0.0	0	0.0	23/24	95.8	12/12	100.0	22/23	95.7	11/12	91.7
II 3	23	12	0	0.0	0	0.0	19/23	82.6	10/12	83.3	16/19	84.2	10/12	83.3
III 10	24	11	0	0.0	1	8.3	20/24	83.3	10/11	90.9	16/20	80.0	9/10	90.0
IV 30	24	12	0	0.0	0	0.0	21/24	87.5	11/12	91.7	19/21	90.5	10/11	90.9

Statistically significant difference from control: *p<0.05
a Includes growth, gestation, lactation and rest periods
b Number of females showing evidence of mating: plug and/or sperm and/or pregnancy
c Number of males for which mating was confirmed in at least one female
d Number of males mated with at least one female for which parturition was evident

Mean gestation length was comparable between the control and treated groups for each pregnancy interval in each generation. Over the entire study no consistent, dose-related effect was seen in mating, fertility or pregnancy indices to indicate an adverse effect of treatment.

Table B.6.6.1/11-03: Summary of survival and growth of offspring

Group g/kg/day	Mean Gestation Length Days	Mean No. Pups at Birth Live	Dead	Total	Pup Viability Index at Birth Live/Total Born No.	%	Mean No. Pups Weaned/ Litter	Postnatal Offspring Survival 0-4 Days	No.	%	4-21 No.	%	Index of Litters Weaned[b] No.	%	Mean Weights of Live Offspring - (grams) Day: 0	4	21
								$F_{0 \rightarrow} F_{1a}$									
I 0	22.1	11.5	0.1	11.6	218/220	99.1	10.7	210/218	96.3	192/195[c]	98.5	19/19	100.0	6.0	9.9	41.1
II 3	21.8	12.8	0.1	12.9	268/271	98.9	12.4	251/268	93.7	247/251	98.4	20/21	95.2	5.8	9.3	37.7
III 10	21.8	12.3	0.3	12.5	196/200	98.0	11.9	194/196	99.0	192/194	99.0	16/16	100.0	5.9	9.4	39.7
IV 30	21.8	11.6	0.1	11.7	221/222	99.5	11.3	217/221	98.2	215/217	99.1	19/19	100.0	6.0	9.6	39.2
								$F_{0 \rightarrow} F_{1b}$									
I 0	22.0	11.7	0.2	11.9	223/226	98.7	11.3	218/223	97.8	215/218	98.6	19/19	100.0	6.1	9.9	40.9
II 3	21.8	12.2	0.6	12.8	232/243	95.5	11.4	223/232	96.1	206/223	92.4**	18/19	94.7	6.1	9.7	43.2
III 10	22.0	12.8	0.3	13.1	153/157	97.5	10.9	145/153	94.8	120/145	82.8**	11/12	91.7	5.8	9.0	37.9
IV 30	21.9	12.6	0.3	12.8	226/231	97.8	11.4	225/226	99.6	194/214[d]	90.7**	17/17[e]	100.0	6.2	9.9	36.9

Significantly different from control: *p≤0.05; **p≤0.01.
a Total number of live pups at birth/Total number of pups (live and dead) at birth.
b Total number of females with litter at Day 21/Total number of females with live pups at birth.
c Excludes data for control female No. 202 whose litter gained a pup during the Day 4-21 interval.
d Excludes data for high-dose female No. 806 whose litter escaped from cage on Day 20 of lactation; this litter contained 11 live pups on Day 14 of lactation.
e Excludes litter of female no. 806; pups escaped from cage on Day 20 of gestation.

Group mg/kg/day	Mean Gestation Length Days	Mean No. Pups at Birth Live	Dead	Total	Pup Viability Index at Birth Live/Total Born No.	%	Mean No. Pups Weaned/ Litter	Postnatal Offspring Survival 0-4 Days: No.	%	4-21 No.	%	Index of Litters Weaned[b] No.	%	Mean Weights of Live Offspring - (grams) Day: 0	4	21
								$F_1 \rightarrow F_{2a}$								
I 0	21.9	12.0	0.2	12.2	216/219	98.6	11.7	201/216	93.1	199/201	99.0	17/18	94.4	5.8	9.4	41.0
II 3	21.8	11.8	0.0	11.8	236/236	100.0	11.6	231/236	97.9*	231/231	100.0	20/20	100.0	6.0	9.7	43.4
III 10	21.9	12.7	0.0	12.7	216/216	100.0	12.4	214/216	99.1**	211/214	98.6	17/17	100.0	6.0	9.1	39.7
IV 30	22.0	11.5	0.4	11.9	207/214	96.7	11.1	206/207	99.5**	200/206	97.1	18/18	100.0	6.2	9.4	40.3
								$F_1 \rightarrow F_{2b}$								
I 0	21.9	12.4	0.4	12.8	186/192	96.9	11.9	178/186	95.7	178/178	100.0	15/15	100.0	5.9	9.4	41.1
II 3	21.9	12.5	0.4	12.9	187/193	96.9	12.7	166/187	88.8*	165/166	99.4	13/15	86.7	5.7	9.2	41.1
III 10	22.1	13.1	0.2	13.4	184/187	98.4	12.7	181/184	98.4	178/181	98.3	14/14	100.0	5.8	9.5	41.3
IV 30	22.1	11.3	0.3	11.6	147/151	97.4	11.1	144/147	98.0	144/144	100.0	13/13	100.0	6.4	10.3	41.3

Significantly different from control: *p≤0.05; **p≤0.01;
[a]Total number of live pups at birth/Total number of pups (live and dead) at birth.
[b]Total number of females with litter at Day 21/Total number of females with live pups at birth.

Group /kg/day	Mean Gestation Length Days	Mean No. Pups at Birth Live	Dead	Total	Pup Viability Index at Birth[a] Live/Total Born No.	%	Mean No. Pups Weaned/ Litter	Postnatal Offspring Survival 0-4 Days: No.	%	4-21 No.	%	Index of Litters Weaned[b] No.	%	Mean Weights of Live Offspring - (grams) Day: 0	4	21
								$F_2 \rightarrow F_{3a}$								
	21.9	11.7	0.2	11.9	268/273	98.2	11.0	266/268	99.3	254/266	95.5	23/23	100.0	6.0	9.5	37.1
	22.0	11.1	0.9	12.0	222/240	92.5**	10.6	219/222	98.6	190/219	86.8*	18/19	94.7	6.1	9.4	36.8
III 10	21.9	12.6	0.1	12.8	202/204	99.0	11.8	202/202	100.0	188/202	93.1	16/16	100.0	6.0	9.5	37.3
	21.9	11.8	0.3	12.1	200/205	97.6	11.0	198/200	99.0	187/198	94.4	17/17	100.0	6.3	9.8	36.7
								$F_2 \rightarrow F_{3b}$								
	21.9	11.2	0.2	11.5	247/252	98.0	11.3	241/247	97.6	237/241	98.3	21/22	95.5	5.9	8.8	38.1
	21.9	12.3	0.4	12.7	197/203	97.0	12.7	192/197	97.5	191/192	99.5	15/16	93.8	5.8	9.0	39.6
	22.1	13.0	0.1	13.1	208/210	99.0	12.4	202/208	97.1	198/202	98.0	16/16	100.0	6.1	9.4	39.9
	21.9	9.8	0.5	10.4	187/197	94.9	9.9	183/187	97.9	178/183	97.3	18/19	94.7	6.3	9.1	38.5

Significantly different from control: *p≤0.05; **p≤0.01.
[a]Total number of live pups at birth/Total number of pups (live and dead) at birth.
[b]Total number of females with litter at Day 21/Total number of females with live pups at birth.

LITTER DATA

Litter size

Mean litter size data on Day 21 of lactation (weaning) was comparable between the control and treated groups for each litter interval throughout the study.

Sex ratio

Pup sex distributions ratios at Day 0 and 21 were generally comparable between the control and treated groups for each litter interval for each generation. No adverse treatment effect on sex distribution data was evident.

Viability index
The mean numbers of live, dead and total pups at birth and pup viability at birth for each pregnancy interval, were comparable between the control and treated groups for each generation. The litter survival indices were comparable between the control and treated groups for each lactation interval in the F0, F1 and F2 generation. In the F0 generation, postnatal survival indices for Days 0-4 and 4-21 were comparable between the control and treated groups for the first lactation interval (F1a). For the second litter interval of the F0, postnatal survival indices for the Day 0-4 interval were comparable between the control and treated groups. During the Day 4-21 interval, survival indices were significantly lower than control in each treatment group. The increase in pup mortality during this interval (i.e. Days 4-21) was attributed to high pup mortality within one or more litters at each treatment level. In the low-dose group the lower pup survival was attributed to one female that experienced complete litter mortality (litter contained 14 live pups at Day 4). In the mid-dose group, one female died on Day 7 of lactation and all seven pups in her litter died during the Day 4-7 lactation interval. Additionally, three mid-dose litters lost five or more pups from their litters during the Day 4-21 lactation interval. In the high-dose group, one female lost nine of l2 pups during the Day 4-21 lactation interval.

In the F1 and F2 generations postnatal survival indices for Days 0-4 and 4-21 during both litter intervals were considered comparable between the control and treated groups. Some statistically significant differences in these indices were observed between the control and treated groups; however, no trend was evident through successive generations to indicate an adverse effect of treatment.

PATHOLOGY
Necropsy
F0, F1 and F2 generations:
Gross necropsy of parental animals of both sexes did not indicate any adverse effect of treatment.

F1, F2 and F3 offspring:
Gross *post mortem* observations of offspring at weaning (F1a, F2a, F3a, F3b) or post-weaning (F1b, F2b) did not demonstrate an adverse effect of treatment. Likewise, evaluation of dead pups recovered at birth and during the 21–day lactation period did not note a treatment-related effect.

Organ weights
F0, F1 and F2 generations:
Mean organ weight data (absolute and relative to body weights or brain weights) were comparable between the control and treated groups for both males and females from the F0 and F1 generations. Some statistically significant differences were noted between control and treated groups both in mean organ weight data and in the relative weight data; however, no trends were evident within dose levels or through these generations.

In the F2 generation, mean organ weight data (absolute and relative) for the males were comparable between the control and treated groups. In the F2 female group, mean liver/body weight ratios were significantly lower than control in each of the treated groups; however, no clear dose-relationship was apparent. Mean liver/brain weight ratios for the treated F2 females were lower than control; however, these differences from control values were not statistically significant. Mean spleen weights (absolute and relative to brain and body weights) were significantly higher than the control value in the F2 mid-dose female group; however, mean spleen weight data for the low- and high-dose F2 females were comparable to control values. In the absence of an effect on spleen weight in the high-dose Fg female group, the change seen in spleen weight data for the mid-dose females was considered spurious and not biologically meaningful. Other mean organ weight data (absolute and relative to body weight or brain weight) for the treated F2 female groups were considered comparable to control data.

Table B.6.6.1/11-04: Organ and body weights and organ/body weight ratios F0 generation – males

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Testes Wt. (g)	Testes Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean	501.167	2.115	100.0	0.012	0.5695	0.053	2.5061	3.451	163.3602	0.667	31.6410	3.072	145.5501	1.385	65.5561	12.133	575.0503
0	S.D.	36.164	0.087	0.0	0.002	0.1158	0.005	0.3038	0.138	9.2303	0.077	4.3608	0.240	14.1464	0.137	6.6087	1.166	67.2877
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
II	Mean	479.000	2.108	100.0	0.010	0.4957	0.053	2.4983	3.464	164.4156	0.684	32.5250	3.077	145.9192	1.326	62.9774	11.968	567.44
3	S.D.	55.049	0.106	0.0	0.001	0.0494	0.005	0.2085	0.262	10.9108	0.078	3.7976	0.357	14.6320	0.097	4.6422	1.370	55.76
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
III	Mean	494.833	2.100	100.0	0.010	0.4978	0.050	2.3989	3.224	153.6772	0.667	31.6830	3.050	145.1569	1.368	65.0393	12.174	579.09
10	S.D.	58.955	0.078	0.0	0.001	0.0600	0.007	0.2975	0.506	24.0305	0.167	7.3828	0.306	12.6720	0.153	5.9821	1.210	45.62
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
IV	Mean	497.000	2.085	100.0	0.011	0.5346	0.051	2.4700	3.137	150.0964	0.636	30.4200	3.190	152.9507	1.354	65.0346	12.418	595.00
30	S.D.	34.123	0.083	0.0	0.002	0.0884	0.004	0.1569	0.497	21.3385	0.119	5.0344	0.370	16.1028	0.092	5.2117	1.626	70.29
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

No statistically significant differences from control.

Table B.6.6.1/11-05: Organ and body weights and organ/body weight ratios F0 generation – females

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Ovaries Wt. (g)	Ovaries Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean	290.261	1.905	100.0	0.014	0.7142	0.073	3.8302	0.088	4.6265	0.508	26.6462	1.934	101.5958	1.010	53.1079	7.746	408.5232
0	S.D.	29.803	0.140	0.0	0.004	0.1807	0.009	0.3961	0.023	1.0948	0.076	3.2098	0.195	10.3488	0.089	4.1257	0.917	48.3397
	N	23	24	24	24	24	24	24	23	23	24	24	21	21	24	24	23	23
II	Mean	295.609	1.901	100.0	0.014	0.7210	0.070	3.6771	0.085	4.4515	0.538	28.3467	1.953	102.6704	1.009	53.0806	7.940	417.4998
3	S.D.	20.978	0.065	0.0	0.005	0.2867	0.010	0.5503	0.028	1.4775	0.103	5.6088	0.186	9.0498	0.116	6.0273	0.764	37.2858
	N	23	24	24	22	22	24	24	23	23	23	23	22	22	24	24	23	23
III	Mean	293.818	1.954	100.0	0.014	0.7346	0.068	3.4729	0.083	4.2087	0.523	26.7278	1.979	102.5988	1.000	51.1712	7.603	392.9238
10	S.D.	38.700	0.130	0.0	0.004	0.1782	0.011	0.5882	0.025	1.1940	0.140	6.4609	0.191	9.4086	0.109	4.6308	0.820	40.2100
	N	22	22	22	21	21	22	22	21	21	22	22	19	19	22	22	20	20
IV	Mean	285.833	1.925	100.0	0.015	0.7982	0.071	3.6697	0.081	4.2217	0.522	27.0856	1.927	100.4362	1.013	52.6802	7.867	409.2639
30	S.D.	20.233	0.066	0.0	0.005	0.2511	0.010	0.4872	0.025	1.2743	0.083	4.0978	0.156	8.8853	0.101	5.0280	0.773	44.3488
	N	24	24	24	24	24	23	23	24	24	24	24	22	22	23	23	23	23

No statistically significant differences from control.

Table B.6.6.1/11-06: Organ and body weights and organ/body weight ratios F1 generation – males

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Testes Wt. (g)	Testes Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean	569.417	2.048	100.0	0.012	0.5981	0.051	2.5054	3.247	157.5361	0.732	35.7729	3.216	157.3347	1.420	62.4469	14.957	731.195
0	S.D.	49.824	0.093	0.0	0.002	0.0951	0.011	0.4931	0.805	36.3510	0.123	5.9597	0.283	15.6909	0.113	6.4152	1.222	62.353
	N	12	12	12	11	11	12	12	12	12	12	12	12	12	12	12	12	12
II	Mean	591.833	2.084	100.0	0.013	0.6216	0.055	2.6258	3.513	169.1032	0.787	37.8719	3.376	162.1841	1.480	71.1869	15.247	733.275
3	S.D.	79.323	0.114	0.0	0.003	0.1450	0.013	0.6360	0.258	16.2654	0.129	6.4209	0.430	20.6084	0.143	7.7089	2.114	106.553
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
III	Mean	559.667	2.038	100.0	0.013	0.6538	0.048	2.3312	3.173	155.1568	0.860*	42.1321	3.237	158.8129	1.464	71.8329	15.097	742.051
10	S.D.	69.674	0.115	0.0	0.002	0.0802	0.009	0.4035	0.507	20.0754	0.132	5.4467	0.329	13.0697	0.147	6.0521	2.451	122.818
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
IV	Mean	563.000	2.027	100.0	0.012	0.5965	0.049	2.4433	3.036	149.8807	0.722	35.6300	3.148	155.8362	1.444	71.2831	14.857	735.269.
30	S.D.	83.538	0.077	0.0	0.002	0.0947	0.006	0.3773	0.728	35.2698	0.120	5.7982	0.516	28.1811	0.233	11.0913	2.977	155.5462
	N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Significantly different from control: *p≤0.05.

Table B.6.6.1/11-07: Organ and body weights and organ/body weight ratios F1 generation – females

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Ovaries Wt. (g)	Ovaries Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean	301.958	1.887	100.0	0.016	0.8478	0.070	3.7096	0.083	4.3953	0.528	28.1152	1.922	101.8550	0.936	49.6682	8.419	447.2939
0	S.D.	23.901	0.120	0.0	0.003	0.1394	0.013	0.6083	0.024	1.3068	0.089	5.3273	0.192	8.3001	0.084	4.2984	0.974	56.8614
	N	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
II	Mean	314.167	1.958	100.0	0.016	0.8302	0.069	3.5524	0.085	4.3583	0.555	28.4796	1.978	101.1283	0.935	48.0896	8.296	424.5618
3	S.D.	23.478	0.092	0.0	0.003	0.1346	0.010	0.5225	0.027	1.4388	0.080	4.6622	0.199	10.0689	0.203	10.8267	0.696	40.2384
	N	24	24	24	22	22	24	24	24	24	24	24	24	24	24	24	24	24
III	Mean	314.739	1.916	100.0	0.016	0.8191	0.063*	3.2774*	0.084	4.4272	0.562	29.4271	1.933	100.7303	0.997	52.1410	8.230	430.4429
10	S.D.	26.064	0.123	0.0	0.003	0.1300	0.009	0.4183	0.025	1.3973	0.118	6.2932	0.257	10.6216	0.099	5.1316	1.019	52.5837
	N	23	23	23	22	22	23	23	23	23	23	23	23	23	23	23	23	23
IV	Mean	309.727	1.850	100.0	0.016	0.8906	0.064	3.5293	0.078	4.3575	0.520	28.7440	1.968	108.0201	1.047	59.2887	8.313	456.3711
30	S.D.	35.407	0.223	0.0	0.004	0.2520	0.006	0.6092	0.029	2.0153	0.066	7.0137	0.191	18.4492	0.226	28.1258	1.095	88.7114
	N	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22	22

Significantly different from control: *p≤0.05.

Table B.6.6.1/11-08: Organ and body weights and organ/body weight ratios F2 generation – males

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Testes Wt. (g)	Testes Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean:	582.000	2.042	0.3526	0.012	0.0021	0.047	0.0081	3.218	0.5562	0.692	0.1189	3.192	0.5498	1.321	0.2281	17.699	3.032
0	S.D.:	49.455	0.069	0.0238	0.002	0.0002	0.006	0.0010	0.264	0.0618	0.115	0.0174	0.281	0.0421	0.082	0.0196	2.558	0.269
	N:	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
II	Mean:	607.667	2.120	0.3526	0.012	0.0020	0.048	0.0080	3.511	0.5845	0.752	0.1239	3.588	0.5914	1.437	0.2372	18.855	3.089
3	S.D.:	71.565	0.100	0.0375	0.002	0.0004	0.012	0.0021	0.354	0.0866	0.119	0.0150	0.473	0.0441	0.181	0.0222	3.339	0.256
	N:	12	12	12	11	11	12	12	12	12	12	12	12	12	12	12	12	12
III	Mean:	576.364	2.045	0.3596	0.013	0.0022	0.041	0.0072	3.280	0.5770	0.762	0.1326	3.421	0.5972	1.455	0.2539*	17.240	2.9911
10	S.D.:	71.813	0.114	0.0456	0.002	0.0002	0.010	0.0020	0.257	0.0837	0.120	0.0165	0.425	0.0672	0.171	0.0271	2.388	0.165
	N:	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
IV	Mean	581.083	1.984	0.3476	0.011	0.0018	0.048	0.0083	3.083	0.5385	0.724	0.1258	3.358	0.5782	1.369	0.2357	17.136	2.954
30	S.D.:	84.951	0.072	0.0474	0.002	0.0003	0.008	0.0015	0.307	0.0727	0.098	0.0162	0.600	0.0576	0.213	0.0175	2.720	0.268
	N:	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Significantly different from control: *p≤0.05.

Table B.6.6.1/11-09: Organ and body weights and organ/body weight ratios F2 generation – males

Group mg/kg/day		Terminal Body Wt. (g)	Brain Wt. (g)	Brain Ratio %	Pituitary Wt. (g)	Pituitary Ratio %	Adrenals Wt. (g)	Adrenals Ratio %	Ovaries Wt. (g)	Ovaries Ratio %	Spleen Wt. (g)	Spleen Ratio %	Kidneys Wt. (g)	Kidneys Ratio %	Heart Wt. (g)	Heart Ratio %	Liver Wt. (g)	Liver Ratio %
I	Mean:	322.375	1.877	100.0	0.016	0.8443	0.064	3.4154	0.082	4.3639	0.503	26.8358	2.003	106.6900	0.983	52.4357	11.231	598.6
0	S.D.:	28.306	0.073	0.0	0.004	0.1974	0.011	0.5786	0.022	1.2049	0.056	3.1484	0.207	9.8944	0.070	3.9449	1.368	70.7
	N:	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
II	Mean:	330.739	1.905	100.0	0.016	0.8112	0.063	3.3202	0.085	4.4476	0.521	27.4079	1.987	104.3400	0.990	51.9983	10.675	561.2
3	S.D.:	41.423	0.133	0.0	0.003	0.1463	0.011	0.4429	0.020	1.0745	0.077	4.0159	0.310	14.2791	0.121	5.6102	1.330	65.9
	N:	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
III	Mean:	327.250	1.878	100.0	0.015	0.8153	0.063	3.3396	0.101	5.3584	0.612**	32.6586**	2.051	109.3628	1.023	54.5466	10.676	570.0
10	S.D.:	29.810	0.096	0.0	0.003	0.1420	0.011	0.6055	0.039	1.9985	0.102	5.4922	0.277	14.4133	0.121	6.4076	1.106	67.0
	N:	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24	24
IV	Mean	322.042	1.883	100.0	0.015	0.7946	0.065	3.4412	0.073	3.8671	0.502	26.5609	2.014	106.7126	0.985	52.2909	10.572	560.8250
30	S.D.:	36.080	0.101	0.0	0.004	0.1732	0.021	1.0691	0.024	1.2543	0.106	4.6991	0.302	13.5043	0.121	5.5482	1.490	71.1884
	N:	24	24	24	24	24	23	23	23	23	24	24	24	24	24	24	24	24

Significantly different from control: **ps0.01.

F3b offspring:
Mean organ weight data (absolute and relative to body weights or brain weights) were comparable between the control and treated groups for both males and females. No treatment-related effect was evident in organ weight data for the F3b offspring.

Histopathology
In total 160 male and female rats (40 adults of each generation F0, F1 and F2 and 40 weanlings of F3b) were examined microscopically. No microscopic findings were considered treatment related. Proliferative tissue changes diagnosed as neoplasms were few. The microscopic tissue alterations, neoplastic and non-neoplastic, were indicative of common incidental histological findings.

CONCLUSION BY STUDY AUTHOR
No treatment-related effect was evident in adult mortality data, body weight and food consumption data, and in-life physical observation data throughout the study (F0, F1 and F2 generation). Throughout the study no consistent effect was seen in mating indices, fertility indices (male) or pregnancy rate data to suggest an adverse effect of treatment. No adverse effects of treatment were evident in maternal weight data, gestation length, parturition data or litter survival indices throughout the study.

Concerning the offspring, no treatment-related effects were indicated in sex distribution data, body weights, survival or gross postmortem findings. Likewise, no effect of treatment was evident in mean organ weight data for randomly selected Day 21 F3b offspring.

No adverse effect of treatment was evident in organ weight data for the F0, F1 generations and F2 adult males. Gross postmortem evaluations of the adult generations and histological evaluations of tissues from randomly selected F0, F1 and F2 high-dose animals and F3b high-dose offspring did not reveal a treatment-related effect.

Assessment and conclusion by applicant:
The oral administration of glyphosate to rats by dietary admixture at a maximum dose level of 30 mg/kg bw/day for three successive generations of CD rats resulted in no treatment-related signs of toxicity in parental animals or offspring. Therefore, the NOAEL for reproduction and offspring is 30 mg/kg bw/day, corresponding to the highest dose tested.

Assessment and conclusion by RMS:
Glyphosate was given in the diet at dose levels of 0, 3, 10 and 30 mg/kg bw/day to CD rats over three generations. Each generation (F0, F1 and F2) consisted of 12 male and 24 female CD rats. The control group received plain diet only. Each parent generation was mated to produce two litters. Parameters evaluated for each generation included: mortality, body weight, food consumption, clinical observation, maternal body weights (gestation/lactation) reproduction-fertility indices (mating, pregnancy and fertility indices), litter data at parturition and organ weight data. Offspring from each litter interval were evaluated during a 21-day lactation period for growth, survival, sex distribution data and gross post mortem observations including organ weight data (F3b offspring only). There were no adverse effects of treatment observed in any of the parameters investigated.

The study is not acceptable (dose levels tested much too low). An effect dose was not reached.

Following deviations from the OECD TG 416 (2001) were observed:
(i) parental animals were dosed 63 days (males) before the mating period (the guideline recommends that dosing shall be continued for at least 10 weeks before the mating period)
(ii) animals were mated in a sex ratio of 1 male:2 females to produce the F1 litters (the guideline recommends that each female shall be placed with a single male from the same dose level (1:1 mating))
(iii) dose levels tested much too low (the guideline recommends that the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering)
(iv) no information on pre-mating dosing period
(ix) oestrous cycle monitoring not performed
(x) pre-coital interval not recorded
(xii) no sperm analyses
(xiii) a quantitative evaluation of primordial follicles not conducted
(xiv) physical and sexual maturation not investigated in offsprings
(xv) thymus of pups not weighed

B.6.6.2. Developmental toxicity studies

B.6.6.2.1. Rat

B.6.6.2/01

Data point	EU data requirement No. CA 5.6.2/001
Report author	[redacted]
Report year	2002 (study report 1996, amendment 2002)
Report title	Glyphosate Acid: Developmental Toxicity Study in the Rat (Including Amendment 001 to Glyphosate Acid: Developmental Toxicity Study in the Rat)
Report No.	[redacted]/P/4819
Document No.	M-301383-01-1
Guidelines followed in study	OECD 414 (2001), OPPTS 870.3700 (1998), 2004/73/EC B.31 (2004)
Deviations from current test guideline	Stated by applicant: Duration of treatment was shorter than required (days 7-16 rather than day 5 post mating to the day prior to scheduled caesarean section). The following endpoints were not assessed: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant, deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414.
Previous evaluation	Yes, evaluated and accepted in the RAR (2015).
GLP/Officially recognised testing facilities	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material:	Glyphosate acid Technical
Lot/Batch:	P24
Purity/Radiochemical purity:	95.6% w/w a.i
Vehicle:	Deionised water

Glyphosate acid was administered in deionised water to groups of 24 Alpk:APfSD Wistar-derived female rats once daily from days 7 - 16 (inclusive) of gestation. The day when spermatozoa were detected in vaginal smears was designated day 1 of gestation. Doses were selected based on the results from a dose-range finding study with 1000 mg/kg/day as the limit dose.

The body weight of animals were recorded on arrival and thereafter on days 4, 7 - 16 (inclusive) and on days 19 and 22 of gestation and the food consumed by each animal over three day periods was calculated from the residues on days 4, 7, 10, 13, 16, 19 and 22, respectively. All animals were observed on arrival and subsequently at least twice each day for changes in behaviour or clinical condition.

Animals were terminated on day 22 of gestation and subjected to postmortem examination. In case there were any uncertainty regarding evidence of implantation, the uterus was removed and stained with ammonium polysulphide to determine whether or not implantation had occurred. The intact gravid uterus (minus ovaries and trimmed free of connective tissue) of pregnant rats was removed and weighed and the ovaries and uterus were then examined for the following:

- Number of corpora lutea in each ovary
- Number and position of implantations subdivided into:
  - live foetuses
  - early intra-uterine deaths (decidual or placental tissue only)
  - late intra-uterine deaths (embryonic/foetal tissue plus placental tissue)
- Individual foetal weights
- Percentage pre-implantation and percentage post-implantation loss

Each foetus was subjected to an external examination as well as an examination of the oral cavity. Furthermore, all foetuses were examined for visceral abnormalities, sexed, eviscerated and fixed in 70 % industrial methylated spirits. After approximately 24 hours the head of each foetus was cut along the fronto-parietal suture line and the brain was examined for macroscopic abnormalities. The carcasses were then further processed, stained, and examined for abnormalities and the degree of ossification. The individual bones of the manus and pes were assessed according to a scale with six grades. The observations were classified as major (permanent structural or functional deviations considered likely to be incompatible with survival or rarely seen) or minor defects or variants (small, generally transient deviations considered compatible with survival).

RESULTS

With the exception of one mis-dosed dam in the control group, all animals survived treatment and there were no clinical signs considered related to treatment, no statistically significant changes in bodyweight or food consumption and no post-mortem findings. There were no differences in the litter data between treated and control animals and no skeletal or visceral findings.

Table B.6.6.2.001-1: Intergroup comparison of maternal body weight (g) (selected timepoints, adjusted means for days 8 and 22)

Glyphosate acid (mg/kg bw/day)				
Day	**0 (control)**	**250**	**500**	**1000**
1	255.6 ± 12.4 N=22	255.5 ± 16.4 N=24	253.5 ± 17.2 N=23	252.8 ± 24.0 N=23
8	288.2	288.1	288.0	287.5
22	406.4	410.1	411.1	408.6

Table B.6.6.2.001-2: Intergroup comparison of maternal performance

Observation	**Glyphosate acid (mg/kg bw/day)**			
	0 (control)	**250**	**500**	**1000**
# Animals Assigned (Mated)	24	24	24	24
# Animals Pregnant	22	24	23	24
#Pregnancy status not determined (intercurrent death)	1	0	0	0
Gravid uterus weight (g) (mean + S D.)	89.7+14.6	87.2+19.3	91.3+15.9	89.9+18.3
Corpora Lutea/Dam (mean + S D.)	15.8+1.2	15.7+1.4	15.5+1.4	15.5+1.3
Implantations/Dam (mean + S D.)	14.4+1.2	12.9+3.6*	14.1+2.1	13.6+2.7
Totally resorbed at termination	0	0	0	1
Total # Litters (viable)	22	24	23	23
Live Foetuses/Dam (mean + S D.)	12.9+2.4	12.4+3.4	13.1+2.7	12.9+2.9
Early Intra-uterine	8.7+14.5	3.4+4.8**	6.2+9.8	5.5+8.4

deaths (Proportion of implants affected) (mean + S D.)				
Late Intra-uterine deaths (Proportion of implants affected) (mean + S D.)	1.3+4.7	0.5+1.8	1.6+4.8	0.3+1.3
Litter Weight (g) (mean + S D.)	62.4+10.4	61.2+14.5	64.3+11.5	63.6+13.7
Mean Foetal Weight (g) (mean + S D.)	4.86+0.29	5.02+0.33	4.95+0.29	4.96+0.27
Proportion of male foetuses	51.9	54.1	53.3	51.0
Pre-implantation loss (%)	8.7	18.0 **	8.8	12.0
Post-implantation loss (%)	9.9	4.0**	7.8	5.8*

* Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

Table B.6.6.2.001-3: Intergroup comparison of foetal defects and variants

Observation	**Glyphosate acid (mg/kg bw/day)**			
	0 (control)	**250**	**500**	**1000**
Major External VISCERAL DEFECTS				
Prop. of foetuses affected (% mean + S.D.)	1/284 (0.3+1.4)	0/297 (0.0+0.0)	1/301 (0.4+1.9)	2/296 (0.8+2.6)
Prop. of litters affected	1/22	0/24	1/23	2/23
Minor External VISCERAL DEFECTS				
Prop. of foetuses affected (% mean + S.D.)	2/284 (0.6+2.0)	2/297 (0.6+2.0)	4/301 (1.4+3.0)	1/296 (0.3+1.6)
Prop. of litters affected	2/22	2/24	4/23	1/23
EXTERNAL VISCERAL VARIANTS				
Prop. of foetuses affected (% mean + S.D.)	27/284 (9.8 + 11.3)	25/297 (8.8+12.1)	24/301 (7.1+7.5)	17/296 (6.1+11.0)
Prop. of litters affected	12/22	11/24	13/23	9/23
Major SKELETAL DEFECTS				
Prop. of foetuses affected (% mean + S.D.)	1/284 (0.3+1.4)	1/297 (0.3+1.5)	1/301 (0.4+1.9)	0/296 (0.0+0.0)
Prop. of litters affected	1/22	1/24	1/23	0/23
Minor SKELETAL DEFECTS				
Prop. of foetuses affected (% mean + S.D.)	60/284 (21.1+14.5)	55/297 (18.7+13.6)	60/301 (20.2+11.0)	51/296 (17.1+12.5)
Prop. of litters affected	18/22	21/24	21/23	20/23
SKELETAL VARIANTS				

Prop. of foetuses affected (% mean + S.D.)	208/284 (72.8+19.4)	199/297 (66.3+18.4)	200/301 (64.9+24.6)	202/296 (67.0+21.1)
Prop. of litters affected	22/22	24/24	23/23	23/23

* number affected/number examined

Situs inversus totalis was observed in 1/296 high dose foetuses and hydroureter/kinked ureter in another. However, these were considered incidental and not related to treatment.

Table B.6.6.2.001-4: Incidence of major defects

Observation	**Glyphosate acid (mg/kg bw/day)**			
	0 (control)	**250**	**500**	**1000**
Encephalocele	1	0	0	0
Extra arch between 12th and 13th right thoracic vertebrae	0	1	0	0
Polydactyly (left hindpaw)	0	0	1	0
Hydroureter	0	0	0	1
Situs inversus totalis	0	0	0	1

Assessment and conclusion by applicant:
This study performed according to OECD 414 (2001) in rats, the NOEL for maternal and developmental effects was 1000 mg/kg bw/day based on no evidence of maternal or developmental toxicity attributable to glyphosate acid at any dose level.

Assessment and conclusion by RMS:
The RMS agrees with the conclusion above. The previous evaluation is thus confirmed.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.

B.6.6.2/02

Data point	EU data requirement No. CA 5.6.2/002
Report author	[redacted]
Report year	1995
Report title	HR-001: Teratogenicity Study in Rats
Report No.	[redacted] 94-0152
Document No.	M-301383-01-1
Guidelines followed in study	Guidelines followed in study Japan MAFF Guidelines 59 NohSan No.4200, 1985 U.S. EPA FIFRA Guidelines Subdivision F, 83-3, 1984 OECD Guideline 414, 1981
Deviations from current test guideline	Stated by applicant: Duration of treatment was shorter than required. The following endpoints were not assessed: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant, deviations from the

	current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414. Day 0 was not defined.
Previous evaluation	Yes (in RAR 2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material: HR-001
Lot/Batch: 940908-1
Purity/Radiochemical purity: 95.68 % w/w a.i
Vehicle: Purified water with 0.5 % sodium carboxymethylcellulose (CMC)

Glyphosate acid was administered in 0.5 % aqueous solution of sodium carboxymethylcellulose to groups of 24 copulated Crj:CD (SD) female rats per group at dose levels of 0, 300 or 1000 mg/kg bw/day from days 6 to 15 of gestation. Doses were selected based on the results of a dose-range finding study.

The body weight of animals were recorded on arrival days 0 (day on which evidence of copulation was recorded), 6-15 (daily during the dosing period) and 20 of gestation and the food consumed by each animal was determined at intervals of days 0-6 and then with four-day intervals. All animals were observed daily during pre-dosing and post-dosing periods and at least twice daily during the dosing period. Animals were terminated on day 20 of gestation and subjected to postmortem examination. In case no uterine implants were clearly apparent, the uterus was stained to detect very early resorptions. The intact gravid uterus of pregnant rats was weighed, and the ovaries and uterus were then examined for the following:

- Number of corpora lutea in each ovary
- Resorbed embryos or dead foetuses subdivided into implantation sites, placental remnants or macerated foetuses (including dead foetuses) according to developmental stage in which resorptions or deaths occurred.
- Number of live foetuses and percent incidences of resorptions and foetal deaths
- Sex ratio, foetal body weights and placental weights
- Foetal examinations: external, visceral and skeletal abnormalities

Foetuses were sexed and the eyes were examined for alterations after removing the palpebral skin. Even-numbered foetuses were examined for visceral abnormalities whereas odd-numbered foetuses were fixed in 70% alcohol, eviscerated, stained and examined for skeletal abnormalities.

RESULTS

All animals survived treatment and there were no clinical signs considered related to treatment except for a statistically significant increased incidence of loose stool in 20 out of 22 pregnant high-dose females.

Table 6.6.2.002-1: Observed clinical signs during the dosing period (gestation days 6 through 15

Clinical sign	**Glyphosate technical (mg/kg bw/day)**			
	0 (control)	**30**	**300**	**1000**
No. of animals examined	23 (1)	24 (0)	24 (0)	22 (2)
No abnormalities detected	23 (1)	23 (0)	22 (0)	2 (0)
Loose stool	0 (0)	0 (0)	0 (0)	20 (2)***
Hair loss	0 (0)	1 (0)	2 (0)	0 (0)
Scabs	0 (0)	0 (0)	1 (0)	0 (0)

Figures represent the number of animals which showed clinical signs during each period of the study.
Figures in parentheses represent the number of animals non-pregnant.

*** Significantly different from control at p<0.001.

Table 6.6.2.002-2: Observed clinical signs during the post-dosing period (gestation days 16 through 20)

Glyphosate technical (mg/kg bw/day)				
Clinical sign	**0 (control)**	**30**	**300**	**1000**
No. of animals examined	23 (1)	24 (0)	24 (0)	22 (2)
No abnormalities detected	23 (1)	23 (0)	22 (0)	13 (2)
Loose stool	0 (0)	0 (0)	0 (0)	9 (0)***
Hair loss	0 (0)	1 (0)	2 (0)	2 (0)
Scabs	0 (0)	0 (0)	0 (0)	0 (0)

Figures represent the number of animals which showed clinical signs during each period of the study.
Figures in parentheses represent the number of animals non-pregnant.
*** Significantly different from control at p<0.001.

Table 6.6.2.002-3: Observations at cesarean section of maternal rats

Observation		Glyphosate technical (mg/kg bw/day)		
	0 (control)	**30**	**300**	**1000**
# Animals Assigned (Mated)	24	24	24	24
# Animals Pregnant	23	24	24	22
#Pregnancy status not determined (intercurrent death)	0	0	0	0
Gravid Uterine Weight (g) (mean ± S.D.)	78 ± 23	85 ± 12	85 ± 19	87 ± 12
Placental weight (mg) (mean ± S.D.)	516 ± 88	511 ± 93	497 ± 62	491 ± 42
No. of corpora lutea (mean ± S.D.)	16.4 ± 2.8	17.2 ± 1.1	17.4 ± 1.7	17.5 ± 1.5
No. of implantations (mean ± S.D.)	14.8 ± 4.4	16.1 ± 1.7	16.1 ± 2.5	16.8 ± 1.4
No. of Live Foetuses	13.7 ± 4.1	15.0 ± 2.1	14.9 ± 2.8	15.4 ± 2.1
% of Foetal resorption and death	7.0	6.8	7.4	8.4
Males Mean Foetal Weight (g) ± S.D.	3.58 ± 0.41	3.60 ± 0.23	3.51 ± 0.43	3.64 ± 0.18
Females Mean Foetal Weight (g) ± S.D.	3.35 ± 0.28	3.39 ± 0.36	3.37 ± 0.36	3.41 ±0 18
Sex Ratio	0.49	0.515	0.461	0.445
Pre-implantation Loss (%)	8.7	18.0**	8.8	12.0
Post-implantation Loss (%)	9.9	4.0**	7.8	5.8*

Statistically significant difference from control group mean, p<0.05 (Student's t-test, 2-sided)
** Statistically significant difference from control group mean, p<0.01 (Student's t-test, 2-sided)

The only external malformations observed were short tail in one low-dose foetus and microphthalmia in one high-dose foetus. Visceral examination revealed two types of malformations: right aortic arch in a foetus of the 300 mg/kg group and ventricular septal defects in one foetus each in the 300 and 1000 mg/kg groups. Visceral variations were observed in all groups including the control group and included thymic remnant in the neck, dilatation of the renal pelvis and left umbilical artery. Skeletal examination revealed three types of malformations: splitting of the ossification centers of the thoracic vertebral bodies in 2, 1 and 2 foetuses in the

control, 300 mg/kg and 1000 mg/kg groups, respectively, asymmetry of the sternebrae with sterno-costal joint displacement in a foetus of the 300 mg/kg group, and fusion of the sternebrae in a foetus of the 1000 mg/kg group. Skeletal variations were observed in all groups including the control group and include cervical ribs shortening of the 13th ribs, lumber ribs, sacralization of the lumber vertebra and asymmetry and/or splitting of the sternebrae. Although the total number of foetuses with skeletal malformations and variation was slightly higher in high dose animals compared to controls, the overall incidences were low and based on the overall pattern considered incidental. According to the study report the Fisher's exact probability test was used for the data on the incidences of maternal rats having fetuses with malformations and variations, incidences of fetal malformations and variations and no statistically significance for changes was reported.

The study was assessed by RAC1 in 2017 concluding that "that no evidence of developmental toxicity was reported in this study."

Table 6.6.2.002-4: Incidence of foetal malformation and variations

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**250**	**300**	**1000**
EXTERNAL malformations				
No. of foetuses examined	314	361	358	339
Microphthalmia	0	0	0	1
Short tail	0	1	0	0
Total No. of foetuses malformed	0	1	0	1
VISCERAL malformation				
No. of foetuses examined	150	174	173	164
Right aortic arch	0	0	1	0
Ventricular septal defects	0	0	1	1
Total No. of foetuses malformed	0	0	2	1
VISCERAL variations				
No. of foetuses examined	150	174	173	164
Thymic remnant in the neck	18	26	24	16
Dilatation of the renal pelvis	1	2	1	2
Left umbilical artery	2	0	3	2
Total No. of foetuses with variations	21	28	28	20
SKELETAL malformations				
No. of foetuses examined	164	187	185	175
Splitting of ossification centers of the thoracic vertebral bodies	2	0	1	2
Fusion of the sternebrae	0	0	0	1
Asymmetry of the sternebrae with sterno-costal joint	0	0	1	0

1 RAC opinion CLH-O-0000001412-86-149/F

displacement				
Total No. of foetuses malformed	2	0	2	3
SKELETAL variations				
No. of foetuses examined	164	187	185	175
Cervical ribs	0	0	1	0
Shortening of the 13th ribs	1	0	1	0
Lumbar ribs	4	1	3	11
Sacralisation of the lumbar vertebra	0	0	1	0
Asymmetry and/or splitting of sternebrae	4	5	5	3
Total No. of foetuses with variations	9	6	11	14

Assessment and conclusion by applicant:
This study using rats performed according to OECD 414 resulted in a NOAEL for maternal effects of 300 mg/kg bw/day based on slightly loose stool at the 1000 mg/kg bw/day dose level. The NOAEL for developmental toxicity was considered to be 1000 mg/kg bw/day. Teratogenic effects were not observed.

Assessment and conclusion by RMS:
The RMS agrees with the conclusion for maternal toxicity and that no teratogenic effects were observed.

The RMS conclusion made in the previous evaluation in 2015 reads:
"*The study is considered acceptable. The evaluation regarding maternal toxicity is agreed, which was confined to loose stool at highest dose level. The NOAEL for developmental toxicity is not supported due to the slight increase in skeletal variations at highest dose level: lumbar ribs were observed in 11 fetuses out of 7 litters compared to only 4 fetuses out of 2 litters in control animals. Teratogenic effects were not observed. Based on findings in dams and foetuses, the NOAEL for both maternal and developmental toxicity is considered to be 300 mg/kg bw/d.*"
The total number of skeletal variations were 9 (5.5%), 6 (3.2%), 11 (5.5%) and 14 (8.0%) in control, low, mid and high dose respectively and differences were not statistically significant. The relation between these observations and treatment is thus considered unclear. Moreover, the information is limited to "lumbar ribs" and the magnitude of the effect is therefore unclear. However since it is listed as a variation rather than a malformation it is assumed that this refers to a not fully formed rib. The increase in lumbar ribs is considered mild and was only observed at the limit dose (4/164 (2.4%), 1/187 (0.5%), 3/185 (1.6%), 11/175 (6.2%)). Taking into consideration that there were no other indication that the substance has an effect on skeletal development and that there were no effects at 1000 mg/kg bw/d in study 5.6.2/001 and study 5.6.2/008, both considered acceptable, this is not considered an effect sufficient for the developmental NOAEL.

Therefore, in contrast to the previous evaluation, the RMS proposes to set the developmental NOAEL at 1000 mg/kg bw/day.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.

B.6.6.2/03

Data point	EU data requirement No. CA 5.6.2/003
Report author	[redacted] *et al.*
Report year	1991
Report title	The effect of glyphosate on pregnancy of the rat (incorporates preliminary investigation)
Report No.	[redacted]43 & 41/90716
Document No.	Not reported
Guidelines followed in study	Guidelines followed in study EPA FIFRA, Subdivision F, \$83-3, OECD No. 414.
Deviations from current test guideline	Stated by applicant: Duration of treatment was shorter than required. The following endpoints were not assessed: uterine weight was not recorded, weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant, deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414. Moreover, the historical control data referred to and further details on the HCD is not included in the report.
Previous evaluation	Yes (study not presented but conclusions from 20002 confirmed in RAR 2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Yes

MATERIALS AND METHODS

Test material:	Glyphosate Technical
Lot/Batch:	206-Jak-25-
Purity/Radiochemical purity:	98.6%
Vehicle:	Purified water with 1% sodium methylcellulose (MC)

Glyphosate was administered in 1% methylcellulose to groups of 24 copulated Crl:CD@ (SD) BR VAF/Plus female rats per group at dose levels 0 (Control), 300, 1000 and 3500 mg/kg bw/day from days 6 to 15 of gestation. The presence of sperm was counted as day 0. Doses were selected based on the results of a dose-range finding study (included as part II in the study report).

Animals were checked daily for signs of reaction to treatment. All animals were weighed initially (Day 1 of pregnancy) and on Days 3, 6, 8, 10, 12, 14, 16, 18 and 20. Water consumption was measured daily from Day 2 through to termination and the mean daily food consumption was measured between the days of recording body weights commencing on Day 3 of pregnancy.

All animals were terminated on Day 20 of pregnancy and examined for congenital abnormalities and macroscopic pathological changes in maternal organs. The ovaries and uteri were examined to determine:

- number of corpora lutea
- number and distribution of live young
- number and distribution of embryofoetal deaths classified as early (only placenta visible at termination) or late: (both placental and embryonic remnants visible at termination).

Uteri or individual uterine horns without visible implantations were further analysed to reveal evidence of embryonic death at very early stages of implantation.

Live foetuses were sexed, externally examined for abnormalities and weighed. Approximately half the foetuses in each litter were examined for visceral abnormalities and the remainder were eviscerated and stained for skeletal examination. In case suspected abnormalities were present, these foetuses were processed with appropriate techniques for further clarification of initial observations. Changes were presented as malformations, anomalies (minor differences from "normal" that are detected relatively frequently either by free-hand sectioning, e.g. increased renal pelvic dilation, or at skeletal examination, e.g. bipartite centrum) or variants.

RESULTS

Preliminary study
Animals received doses of 0 (Control), 500, 1000 and 3500 mq glyphosate/kg bw/day by qavage from Day 6 to Day 15 of pregnancy inclusive. Animals were sacrificed on Day 20, thereafter subjected to postmortem examination, litter parameters were recorded and foetuses were examined for gross abnormalities. Effects noted in high-dose animals included post-dosing salivation (occasionally brown stained), wet coats and noisy respiration in all animals, loose faeces, increased water and reduced food consumption throughout the treatment period and reduced bodyweight gain Days 6 - 8 and Days 12 - 16. Post-dosing salivation was also observed in all mid-dose animals, occasional brown stained (4/10 animals), noisy respiration (3/10 animals), slight reduction in food consumption throughout the treatment period and reduced bodyweight gain Days 6 - 8 and Days 12 - 14. In low-dose animals a slightly reduced bodyweight gain on Days 6 - 8 and Days 12 – 14 were noted. Foetal effects were limited to a slight reduction in litter and mean foetal weight in the high-dose group.

Main study
One high-dose female was sacrificed on Day 10 immediately after dosing probable due to an intubation error (the post-mortem examination showed cloudy white fluid in the thoracic cavity. Two additional females were sacrificed on Day 7 and 13 respectively following signs of respiratory distress (noisy respiration/gasping). The cause of respiratory distress could not be clarified by the post-mortem examination but considering that these signs, although less severe, were observed among high-dose animals, these two deaths are considered to be related to treatment. Clinical signs observed in treated high-dose animals included post-dose salivation in all with wet coats in approximately half of the animals. Loose faeces were observed from Day 7 or 0 of pregnancy and persisted throughout the treatment period. Noisy respiration and/or gasping were observed in 17/22 females on one or more occasions. Noisy respiration was also observed in two mid-dose females.

Table 6.6.2.003-1: Summary of maternal performance and clinical signs

Clinical sign		**Glyphosate technical (mg/kg bw/day)**		
	0 (Control)	**300**	**1000**	**3500**
No. of animals in group	25	25	25	25
Non-pregnant	2	2	0	0
Sacrificed	0	0	0	3
With live young on Day 20	23	23	25	22
Clinical signs				
Salivation, occasionally	0	0	0	22
Loose faeces	0	0	0	22
Noisy respiration	0	0	2	15
Wet coats	0	0	0	13
Gasping	0	0	0	5
Area of hair loss/scabbing	2	3	3	0

In high dose animals, water consumption increased following start of treatment and continued to increase throughout the remainder of the treatment period. Thereafter, intake decreased, but was still slightly greater than controls at termination. Food consumption was decreased during the treatment period but comparable to controls to termination. This was reflected in the bodyweight gain which was markedly reduced during the first two days of treatment when compared to the concurrent control values. The bodyweight gain was reduced during the

entire treatment period and also during the post-dosing period and until termination on day 20. Data was not statistically analysed.

Table 6.6.2.003-2: Mean body weights (g) and body weight gain (g) relative to start at treatment on Day 6

Glyphosate technical (mg/kg bw/day)				
Parameter	**0 (control)**	**300**	**1000**	**3500**
Mated females	25	25	25	25
No of animals included in assessment	23	23	25	22
Body weights				
Day 1	194.5	196.6	196.9	199.6
Day 3	221.8	223.1	224.2	227.8
Day 6	250.5	254.0	253.3	257.8
Day 8	266.0	270.1	266.6	260.3
Day 10	282.6	287.0	283.6	275.0
Day 12	302.0	306.4	302.9	292.9
Day 14	319.1	324.5	319.1	303.4
Day 16	344.0	347.3	343.9	321.5
Day 18	376.5	378.2	374.9	354.8
Day 20	414.4	413.6	405.4	390.5
Body weight gain relative to Day 6				
Day 1	-56.0	-57.4	-56.4	-58.2
Day 3	-28.7	-30.9	-29.2	-30.0
Day 6	0.0	0.0	0.0	0.0
Day 8	15.5	16.0	13.3	2.5 (↓84%)
Day 10	32.1	33.0	30.3	17.2 (↓44%)
Day 12	51.5	52.4	49.6	35.1 (↓32%)
Day 14	68.7	70.5	65.8	45.5 (↓34%)
Day 16	93.6	93.3	90.6 (↓3%)	63.7 (↓32%)
Day 18	126.0	124.1	121.6	97.0 (↓23%)
Day 20	163.9	159.5	152.1 (↓7%)	132.6 (↓19%)

There were no significant changes in implantation rate, post-implantation loss, litter size or sex ratio. There was an apparent increase in pre-implantation loss in high-dose animals (also observed in the preliminary study) which was not considered treatment-related by the study author since treatment begins after implantation. The litter and mean foetal weights were reduced in high-dose foetuses and the reduction in mean foetal weight was statistically significant.

Table 6.6.2.003-3: litter data – group mean values

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
# Animals assigned (mated)	25	25	25	25
# Animals with live young on Day 20	23	23	25	22
#Pregnancy status not determined (intercurrent sacrifice)	0	0	0	3
Mean no. of corpora lutea	16.3	15.5	16.4	16.9
Mean no. of implantations	14.6	13.6	14.0	13.6
Pre-implantation loss (%)	10.0	13.1	14.6	19.3** (↑93%)

% of foetal resorption and death (post implantation loss)	6.1	7.3	5.7	3.6
Mean no. of live foetuses/litter	13.7	12.7	13.2	13.1
Mean Foetal Weight (g)	3.96	3.90	3.89	3.71** (↓6%)
Sex Ratio (% male foetuses)	50.9	45.5	47.5	45.5

** Statistically significant difference from control group mean (Kruskal-Wallis H-statistic followed, if significant, by

There were no significant differences in malformations and visceral anomalies between foetuses in treated groups and control however the incidences of rib distortion (wavy ribs), presented as a skeletal anomaly, was markedly increased in high-dose animals and marginally higher in mid-dose animals. The incidences of other skeletal effects presented as anomalies such as reduced ossification (table 6.6.2.003-4) were higher in all treated groups compared to the concurrent controls but claimed by the study author to be only slightly outside the expected range in comparison with the background control data. However, the historical control data referred to and further details on the HCD is not included in the report. Based on the background incidence and the lack of a clear dosage-response, the study author did not consider that these skeletal changes could be ascribed treatment. The RMS disagrees with this line of reasoning since, as seen in table 6.6.2.003-4, the incidence of foetuses with skeletal anomalies increased with dose and was statistically significant in mid and high dose animals. Moreover, the incidence in high dose animals (35.7 %) was 8,5-13.8 % higher than the claimed background range (21.9-27.2%). However, the bodyweight gain in high-dose animals during treatment (i.e. days 6-16) was much lower in high dose dams compared to controls and the mean foetal weight was 6% lower at birth. The presence of wavy ribs and the other ossification related skeletal anomalies in high-dose animals may thus be considered secondary to the reduced bodyweights whereas similar findings at lower frequencies in the mid-dose group (1000 mg/kg bw/d) may not. Nevertheless, the incidence in mid-dose animals was only 3 foetuses from 2 litters compared to 1 foetus from 1 litter in controls. Moreover, the skeletal effects observed are considered variations rather than malformations and they may be transient effects likely to resolve during the first week of life2.

Table 6.6.2.003-4: Summary of foetal malformations and anomalies

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
Malformations				
No. of foetuses examined	314	292	329	289
Total no. of foetuses with malformations (affected litters)	1 (1))	2 (2)	1 (1)	3 (2)
Mean % malformations	0.3	0.8	0.3	1.1
Background range (% affected foetuses) 0.3 – 1.7				
Visceral anomalies				
No. of foetuses examined	158	147	162	144
Total no. of foetuses (affected litters)	9 (8)	11 (8)	5 (4)	11 (10)
Mean % affected foetuses	5.8	6.9	2.7	7.6
Background range (% affected foetuses)				

2 Discussed in article by CA. Kimmel, MR. Garry and JM. DeSesso in Birth Defects Research (Part B) 101:379–392 (2014) "Relationship Between Bent Long Bones, Bent Scapulae,and Wavy Ribs: Malformations or Variations? The authors are connected to Exponent, Inc., Toxicology and Mechanistic Biology.

4.6 – 5.8				
Skeletal anomalies				
No. of foetuses examined	155	143	166	142
Total no. of foetuses (affected litters)	19 (11)	36 (16)	46 (19)	55 (19)
Mean % affected foetuses	11.7	22.6	28.4*	35.7**
Background range (% affected foetuses) 21.9 – 27.2				

* Statistically significant difference from control group mean (Kruskal-Wallis H-statistic followed, if significant, by intergroup comparison with the control (distribution-free Williams' test, significant at $p<0.05$)

** Statistically significant difference from control group mean (Kruskal-Wallis H-statistic followed, if significant, by intergroup comparison with the control (distribution-free Williams' test, significant at $p<0.01$)

Table 6.6.2.003-5: Summary of foetal skeletal and visceral malformations (group incidences)

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
No. of foetuses examined (no. of litters examined)	314 (23)	292 (23)	329 (25)	289 (22)
No. of foetuses affected (no. of litters affected)	1 (1)	2 (2)	1 (1)	3 (2)
Skeletal and visceral malformations				
Description				
Cranial				
Microphthalmia	0 (0)	1 (1)	0 (0)	0 (0)
Palatine irregularity	0 (0)	0 (0)	0 (0)	2 (2)b,c
Naso-pharangeal fistula	0 (0)	0 (0)	0 (0)	1 (1)b
Cervical				
Multiple vertebral irregularities	0 (0)	0 (0)	0 (0)	1 (1)
Connected 5th to 6th vertebral arches	0 (0)	0 (0)	0 (0)	1 (1)c
Thoracic				
Interventricular septal defect	0 (0)	0 (0)	1 (1)a	1 (1)b
Abdominal/Lumbar				
Marked distension of urinary bladder	1 (1)	0 (0)	0 (0)	0 (0)
Absent cleft of median liver lobe	0 (0)	0 (0)	1 (1)a	0 (0)
Other				
Termination of vertebral column (sacral) - includes anury	0 (0)	1 (1)	0 (0)	0 (0)

Individual foetuses may occur in more than one category, superscripts refer to the same foetus

Table 6.6.2.003-6: Summary of foetal visceral anomalies (group incidences)

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
No. of foetuses examined (no. of litters examined)#	158 (23)	147 (23)	162 (25)	144 (22)
No. of foetuses affected (no. of litters affected)#	9 (8)	11 (8)	5 (4)	11 (10)
Visceral anomalies				
Description				
Subcutaneous/subdural haemorrhage: trunk, tail, limbs, head	3 (3)	3 (3)	1 (1)	0 (0)
Cranial				
Small eye	0 (0)	1 (1)	0 (0)	0 (0)
Moderate haemorrhage anterior chamber of eye	0 (0)	0 (0)	0 (0)	1 (1)
Cervical				
Reduced side of thyroid	0 (0)	0 (0)	0 (0)	1 (1)
Thoracic				
Anomalous cervicothoracic arteries	0 (0)	1 (1)	0 (0)	0 (0)
Interventricular septal defect (small)	1(1)	4(3)	1(1)	2(2)
Abdominal/Lumbar				
Intra-abdominal haemorrhage	0 (0)	0 (0)	2 (2)	1 (1)
Abdominal lobation of liver	2 (2)	2 (2)	0 (0)	4 (3)
Increased dilation of renal pelvis/ureter	3(2)	-(-)	1(1)	1(1)
Displaced testis	0 (0)	0 (0)	0 (0)	1 (1)

Malformed foetuses are excluded

* Individual foetuses may occur in more than one category

Table 6.6.2.003-7: Summary of skeletal anomalies (group incidences)

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
No. of foetuses examined (no. of litters examined)#	155 (23)	143 (23)	166 (25)	144 (22)
No. of foetuses affected (no. of litters affected)#	19 (11)	36 (16)	46 (19)	57 (18)
Description and incidences				
Reduced ossification of one or more cranial centres	3 (3)	2 (2)	12 (8)	10 (5)
Reduced ossification of cervical vertebral	0 (0)	0 (0)	1 (1)	0 (0)

arches				
Reduced ossification of sacro-caudal vertebral arches	3 (2)	8 (6)	17 (11)	15 (10)
Reduced ossification of one or more cantres of pelvic girdle	0 (0)	3 (3)	4 (3)	6 (4)
Cranial				
Area of ossification within suture	0 (0)	1 (1)	1 (1)	0 (0)
Cervical				
Cervical ribs	0 (0)	2 (2)	0 (0)	1 (1)
Thoracic				
Irregular ossification of vertebral centre	2 (2)	1 (1)	3 (3)	3 (3)
Distortion of ribs	1 (1)	0 (0)	3 (2)	28 (11)
Shortened 13th rib(s)	0 (0)	0 (0)	2 (1)	0 (0)
Lumbar				
Lumbar rib(s)	13 (8)	24 (13)	22 (12)	24 (12)
One extra thoracolumbar vertebra	1 (1)	2 (2)	0 (0)	6 (3)

Malformed foetuses are excluded
Individual foetuses may occur in more than one category

Assessment and conclusion by applicant:
In this developmental toxicity study according to OECD 414, the maternal NOAEL was set at 300 mg/kg bw/day based on mortality, clinical signs, decreased body weight gain and reduced food and water intake indicating a clear adverse effect of glyphosate administration at 3500 mg/kg bw/day and a possible impact at 1000 mg/kg bw/day with noisy respiration and decreased body weight gain. There was no evidence of teratogenicity in this study. Secondary to maternal toxitcity, reduced foetal weight, delayed ossification and a lower number of viable foetuses was observed at the extremely high top dose of 3500 mg/kg bw/day and reduced/delayed ossification and increased incidence of skeletal variations were observed at 1000 and 3500 mg/kg bw/day. The developmental NOAEL is considered 300 mg/kg bw/day.

Assessment and conclusion by RMS:
The RMS agrees with the maternal NOAEL. The RMS also agrees that effects seen in high-dose foetuses as reduced foetal weight (6%), delayed ossification and a lower number of viable foetuses may be secondary to the maternal toxicity observed. The increase in skeletal anomalies observed at 1000 mg/kg bw/d with no clear maternal toxicity is considered mild and the relation to treatment is unclear. This is supported also from conclusions in the RAC opinion *"Evidence of delayed ossification, increased incidence of foetuses with wavy ribs and reduced foetal weight was recorded at 1000 mg/kg bw/d (Table below). RAC considers that the effects on fetal weight and on the degree of ossification are secondary effects, due to the maternal toxicity observed in the high dose group and notes that an increase in wavy ribs was not recorded in any of the other available developmental toxicity studies."*

The incidence of reduced ossification of sacro-caudal vertebral arches is increased also in the low dose-group, likewise the increased incidences of lumbar ribs are similar in all treated groups however the NOAEL is considered to be 300 mg/kg bw/d where the overall incidence was yet lower.

It is noted that the top dose 3500 mg/kg bw/d is far above the limit dose of 1000 mg/kg bw/d in OECD TG 414.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. Uterine weight was not recorded.

B.6.6.2/04 / B.6.6.2/05

Data point	EU data requirement No. CA 5.6.2/004 with amendment (establishment of No Observed Adverse Effect Level (NOAEL)) in 5.6.2/005
Report author	[redacted]
Report year	1991
Report title	Teratogenicity study in Wistar rats
Report No.	[redacted].883.TER-R
Document No.	Not reported
Guidelines followed in study	Not stated but considered by the applicant to generally be in accordance with OECD Guideline 414, 1981
Deviations from current test guideline	Stated by applicant: Duration of treatment was shorter than required. The following endpoints were not assessed: weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant the deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414. Additional deviations noted by the RMS: The control group contained 10 more animals than the treatment group.
Previous evaluation	Yes (in RAR 2015) The study was considered "supplementary" in the previous assessment.
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Accepted as supportive information. Treatment stopped day 15 instead of the day prior to terminationon day 20. Furthermore, since the study is limited to one dose level (limit dose), a developmental NOAEL cannot be set.

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: 1071-83-6
Purity/Radiochemical purity: 96.8%
Vehicle: Postman brand refined groundnut (peanut) oil

The dose level used in the main study was based on the results from two dose-range finding studies; one in non-pregnant rats (study I) and one in pregnant rats (study II).

In study I three females/group received doses of 0, 100, 400 or 1000 mg/kg bw/day for 10 days resulting in emaciation in one mid and high-dose animal, urinary incontinence and red nasal discharge in one high-dose animal and slightly decreased bodyweight and feed intake in high-dose animals. In study II five mated rats received 1000 mg/kg bw/d from day 6-15 of gestation. Historical control data from 70 animals was used instead of a concurrent control group. Urinary incontinence was noted in two animals and loose stool was noted in one. The body weight gain was slightly reduced compared to historical controls while feed intake was similar.

Main study
Glyphosate was administered in groundnut (peanut) oil to 25 mated female Wistar rats at dose level of 1000 mg/kg bw/day from days 6 to 15 of gestation. The presence of a vaginal plug or spermatozoa in the vaginal smear was considered day 0. A control group of 35 rats receiving vehicle only was included in the study.
Animals were checked twice daily for signs of reaction to treatment. All animals were weighed initially (Day 0), daily from Day 6 to 15 and on day 20, the day for termination. Feed intake was calculated based on the feed left on Days 6, 11, 16 and 20.

All females were subjected to a gross pathological examination and the uterus along with the ovaries was excised and weighed. The following observations were made:

- uterine weight
- number of corpora lutea
- number of implantations
- embryonic and foetal resorptions.

All foetuses were examined for external malformations, sex and weight and reported as:

- total number of foetuses
- number of dead foetuses
- number of abnormal foetuses
- number of live foetuses (including foetal weight)
- number of male foetuses
- number of male foetuses
- sex ratio

Approximately half of the foetuses in each litter was examined for visceral malformations categorised as normal variants, minor and major malformations. The remaining half of the foetuses were fixed, stained and evaluated for skeletal malformations classified into normal variants, minor and major malformations.

RESULTS

All animals survived treatment and the incidences of clinical signs observed (i.e. weakness and dullness, soft faeces, red coloured nasal discharge, snuffling, lacrimation and urinary incontinency) were similar between treated animals and controls. There were no statistically significant differences in body weight or body weight gain during pre-treatment, treatment, post-treatment, the overall gestation period or in the corrected body weight gain when compared to controls. There were no treatment-related visceral findings at necropsy.

Table 6.6.2.004-1: Mean body weights and corrected body weight gain (g) relative to start at treatment on day 6 (group means ± SD)

Parameter	**Glyphosate technical (mg/kg bw/day)**	
	0 (control)	**1000**
Mated females	35	25
No. of animals included in assessment (pregnant at term)	30	20
Body weights at		
Day 0	186 ± 18.5	188 ± 13.9
Day 6	199 ± 18.2	200 ± 16.5
Day 15	218 ± 19.7	220 ± 17.6
Day 20	258 ± 27.2	253 ± 30.2
Corrected body weight gain (Day 20 bw – uterine weight)	208 ±23.3	210 ± 21.6

The number of corpora lutea, implantations, embryonic and foetal resorptions, pre-implantation and post-implantation loss were similar in both groups and there were no significant differences in litter size, the incidence of dead or abnormal foetuses, foetal body weights or foetal sex ratios.

Table 6.6.2.004-2: Summary of food consumption (group means ± SD)

Observation		Glyphosate technical (mg/kg bw/day)
	0 (control)	**1000**
No. animals assigned (mated)	35	25
No. animals with live young on Day 20	30	20
No. pregnancy status not determined (intercurrent sacrifice)	0	0
No. of corpora lutea	11 ± 1.7	11 ± 2.0
No. of implantations	10 ± 2.5	9 ± 3.2
Pre-implantation loss (%)	38 (13)	35 (20)
No. of embryonic resorptions (%)	24 (8)	19 (11)
No. of foetal resorptions (%)	0 (0)	0 (0)
Post implantation loss (%)	24 (8)	19 (11)
No. of dams with any resorptions (%)	15 (50)	11 (55)
No. of dams with total resorption (%)	0 (0)	0 (0)
Total no. of foetuses	261	157
Mean litter size	9	8
No. of dead foetuses/litter	0	0
No. of abnormal foetuses	0	0
Mean foetal weight (g)	3.6 ± 0.4	3.7 ± 0.3
Foetal sex ratio (male:female)	1:1	1:1.3

The incidence of haemorrhagic spots was slightly higher in the treated group than in the control. However, the difference was not statistically significant and considered a normal variation by the study author. There were no major external findings noted in either of the study groups and no differences in visceral variations or minor/major malformations. None of these observations reached the level of statistical significance. No major visceral malformations were noted.

Table 6.6.2.004-3: Summary of foetal external findings (% foetuses affected)

Glyphosate technical (mg/kg bw/day)		
	0 (control)	**1000**
No. of foetuses examined	261	157
No. of litters examined	30	20
Variations		
Haemorrhagic spot	5.4	10.2
Minor Malformations (categorised as a malformation which would not be expected to directly affect the survival of the fetus)		
Foetus small	0.8	0.6
Snout short	0.4	0.0
Major Malformations		
No. of foetuses with major malformations	0	0

However, the incidence of delayed ossification of caudal vertebral arch, forelimb-proximal phalanx and hindlimb-distal phalanx was significantly higher in the treated group compared to the control. In contrast, the incidence of incomplete to partial ossification of parietal and interparietal of the skull was less in the treated group.

Table 6.6.2.004-4: Summary of foetal findings (% foetuses affected, statistical difference to control by Contingency test in bold)

Observation		Glyphosate technical (mg/kg bw/day)
	0 (control)	**1000**
No. of foetuses examined	140*	83
No. of litters examined	30	20
Major Malformations		
Skull		
Malformed Supraoccipital bone	0.7	0.0
Parietal bone	0.7	2.4
Interparietal bone	2.1	3.6
Temporal bone	0.7	1.2
Basisphenoid	1.4	1.2
Sternebra #6	0.0	1.2
Cervical vertebral arch # 3-7	0.7	0.0
Scapula	0.7	0.0
Incomplete Palatine	0.0	2.4
Zygomatic arch	2.1	0.0
Cervical vertebral arch #5	0.7	0.0
Cervical vertebral arch #6	1.4	0.0
Fused Sternebrae #1-6	0.7	0.0
No. of foetuses with major malformations	17 (12%)	10 (12%)
No. of dams with foetuses with major malformations	8 (27%)	6 (30%)
Normal variants		
delay in ossification of forelimb-proximal phalanx 1 of 2	26.4%	36.1%
delay in ossification of forelimb-proximal phalanx 2 of 2	70.0%	**83.1%**
delay in ossification of hindlimb-distal phalanx 1 of 5	17.9%	13.3%
delay in ossification of hindlimb-distal phalanx 2 of 5	32.1%	31.1%
delay in ossification of hindlimb-distal phalanx 3 of 5	2.9%	7.2%
delay in ossification of hindlimb-distal phalanx 5 of 5	17.1%	**32.5%**
delay in ossification of caudal vertebral arch 1 of 2	22.9%	**39.8%**
delay in ossification of caudal vertebral arch 2 of 2	3.6%	3.6%
incomplete to partial ossification of parietal and interparietal of the skull	87.9%	**56.6%**

* One foetus noted with short snout during visceral examination was also evaluated for skeletal changes

Assessment and conclusion by applicant:
This study (performed according to OECD 414) showed no evidence of maternal toxicity. The incidence of foetal malformations was not increased in the treated group compared to the control. There was limited evidence of a higher incidence of delayed ossification (caudal vertebral arch, forelimb proximal and hindlimb distal phalanges) in the group receiving glyphosate. On the other hand, delayed ossification of other parts of the skeleton, in particular the skull, was more frequently seen in the control group. Thus, there was no clear and

consistent impact of test compound administration on the process of ossification. Thus, the NOAEL for both maternal and developmental toxicity is assumed to be 1000 mg/kg bw/day.

Assessment and conclusion by RMS:
The RMS agrees that the maternal NOAEL is 1000 mg/kg bw/day and that the toxicological significance of the pattern of ossification is unclear and difficult to assess in the absence of additional dose groups. Therefore it is not considered possible to set a NOAEL for developmental toxicity. The previous evaluation is thus confirmed.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.

B.6.6.2/06

Data point	EU data requirement No. CA 5.6.2/006
Report author	[redacted]
Report year	1986
Report title	Report on effect of glyphosate technical of [redacted], India; on reproductive process. Segment II – Teratological study
Report No.	Not reported
Document No.	Not reported
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes. The study was evaluated and **not accepted** (Monograph 2000).
GLP/Officially recognised testing facilities	No (not compulsory at that time)
Acceptability/Reliability	Not acceptable due to several serious reporting deficiencies.

Short description of study design and observations	20 mated female Wistar rats per group were administered glyphosate ([redacted] India; purity not reported) at dose levels of 0 (control, vehicle not stated), 100 and 500 mg/kg bw/day on gestational days 6 through 15 by gavage. Dams were sacrificed and foetuses delivered on day 20.
Short description of results	Neither maternal nor reproduction and embryo-foetal effects were observed up to the highest dose tested. Thus, the NOEL for both maternal and developmental toxicity was 500 mg/kg bw/day in this study.
Reasons for why the study is not considered relevant/reliable or not considered as key study	The notifier has no access to this study report. Since the study was part of the earlier data package available to the former RMS of the active substance glyphosate, the AGG would have to send a "request for administrative assistance" (Art. 39 of Regulation (EC) No. 1107/2009) to the BVL.

Assessment and conclusion by RMS:
The RMS agrees with the applicant's conclusions above. It is noted that the top dose 3500 mg/kg bw/d is far above the limit dose of 1000 mg/kg bw/d in OECD TG 414.

B.6.6.2/07

Data point	EU data requirement No. CA 5.6.2/007 Note from the RMS: This study was also submitted for B.6.6.2/017.
Report author	Anonymous
Report year	1981
Report title	Teratological investigation of glyphosate in rats and rabbits
Report No.	Not stated
Document No.	Not stated
Guidelines followed in study	Not stated
Deviations from current test guideline	Stated by the applicant: Treatment duration was slightly shorter than required; group size smaller than required; missing endpoints: no data on food consumption reported, weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected;, no data on results of foetal or visceral skeletal examination. Additional deviations noted by the RMS: • the laboratory diet was not specified further than "LATI" and "food pellets of 10 x 30-40 nm size". • It is not clearly stated if tha appearance of sperm or vaginal plug was considered day 0. • The nature and frequency of checks for clinical conditions was not described. • Results from the histopathological investigations are not included in the report. • uterine weight was not recorded • number of corpora lutea; • number and percent of pre- and post-implantation losses. • sex ratio was not calculated • fetal body weight by sex
Previous evaluation	Considered supplementary in RAR 2015
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Not considered acceptable by the RMS. A full study summary is yet included for transparency since the study was considered in the assessment by RAC in 2017.

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: Not stated
Purity/Radiochemical purity: 96.8%
Vehicle: Laboratory rodent food

In this study, the developmental toxicity study of glyphosate was investigated in both rats and rabbits.

Rat
The substance was administered groups of time-mated female CFY rats from Days 6 to 18 of gestation in diet. In the control and high dose groups ten additional females were treated and allowed to give birth and litters were kept on study until day 28 post partum ('postnatal group'). Concentrations of glyphosate in the diet were not reported, but the test item intake between Days 6 and18 of gestation was 22, 103 and 544 mg/kg bw/day, respectively, and 558 mg/kg bw/day in high dose postnatal females. Females of the 'prenatal group' were sacrificed on Day 20 of gestation and following opening of the uterine horns, the total number of implantations,

of live, dead and resorbed embryos/foetuses were counted. The mean litter and foetal weights, gestational growth, the mean body weight gain and food consumption were calculated. Live foetuses in the prenatal groups were fixed and examined for visceral and skeletal abnormalities and the heart, lungs, thymus, liver, intestines, pancreas, spine, skeletal muscles, intervertebral discs and nervous tissue were examined histologically. Females of the postnatal group and their offspring were sacrificed on day 28 post partum. Gross and histopathology was conducted on each animal and the heart, lungs, thymus, stomach, liver, kidney, spleen and reproductive organs. were histopathologically examined.

Rabbit
The substance was administered groups of New Zealand White rabbits from Days 6 to 19 of gestation via diet. Concentrations of glyphosate in the diet were not reported, but the test item intake was stated to be 10.5, 50.7 and 255.3 mg/kg bw/day, respectively. Females of the 'prenatal group' were sacrificed on Day 28 of gestation and following opening of the uterine horns, the total number of implantations, of live, dead and resorbed embryos/foetuses were counted. The mean litter and foetal weights, gestational growth, the mean body weight gain and food consumption were calculated. Live foetuses in the prenatal groups were fixed and examined for visceral and skeletal abnormalities and the heart, kidney, spleen and lungs were prepared and fixed. Skeletal investigations were made.

RESULTS

RAT
Treatment with glyphosate had no effect on the maternal organism. Body weight was similar in the treated groups, body weight gain in the mid and high dose group was slightly lower than in controls throughout pregnancy, gross examination and histopathology carried out on foetuses of the 'prenatal group' indicated no changes in any group. In the postnatal group no toxic symptoms or gross pathology alterations were observed in high dose or control females; and no macroscopic or microscopic changes were noted in their offspring.

Based on these results the non-teratogenic level is larger than 544 mg/kg bw/day his st

Table 6.6.2.007-1: Summary of body weight data (group means) - Prenatal group

Glyphosate (mg/kg bw/day)	**0 (control)**	**22**	**103**	**544**
Mated females	13	12	15	15
Body weights (g) at				
Day 6	255	261	280[1]	280
Day 10	275	276	294	295
Day 19	304	309	331	338
Body weight gain day 1-20 (g)	81	85	71	68

[1] No value was given for Day 6, this is the mean for Day 7

Table 6.6.2.007-2: Summary of body weight data (group means) – Postnatal group

Dose Group (mg/kg bw/day)				
Parameter	**0 (control)**	**22**	**103**	**558**
Mated females	10	0	0	10
Day 6 post coitum	266			262
Day 11 post coitum	295			297
Day 16 post coitum	328			338
Day 19 post coitum	310			344
Body weight gain Day 1-21 post coitum (g)	123			117[1]
Day 0/12 post partum	278			293
Day 8/92 post partum	288			311
Day 14/152 post partum	299			313
Day 21/222 post partum	274			305
Day 28 post partum	275			295

[1] in the report, an obviously wrong value of 73 g is given; recalculation resulted in the presented value
[2] weighing day for the control/weighing day for the high dose

Table 6.6.2.007-3: Summary of litter parameters – Prenatal group (strike-through numbers were reported by the applicant however values corrected by the RMS in line with the study report are presented in italics).

Glyphosate (mg/kg bw/day)				
Parameter	**0 (control)**	**22**	**103**	**544**
No. of mated females	13	12	15	15
Mean No. of implantations/dam	12.08	12.58	14.67	13.00
Mean No. of dead foetuses/dam	1.77	0.67	*0.93*	0.67
Foetal loss (%)	14.65	5.30	12.73	5.13
Mean No. of live foetuses/dam	10.31	11.92	12.80	12.33
Mean litter weight (g)	25.46	28.23	28.13	30.53
Mean foetal weight (g)	14.65[1]	5.30[1]	2.07[2]	2.50[2]
Externally abnormal foetuses	0	0	0	0

[1] values as given in the report, but unrealistic for rat foetuses at term, in looking at the table in the report it appears the values for foetal loss (%) were accidently repeated here.
[2] values as given in the report, but untypically low for rat foetuses at term

RABBIT

According to the study report, treatment had no effects on behaviour and appearance however there is no data in the original report to support this statement. Bodyweights were higher in treated animals during gestation (i.e. 426, 452, 516 and 578 g in control, low, mid and high dose animals, respectively. According to the study report the gestational body weight was slightly lower in treated groups compared to controls however this is not clear from the data available. The data available do however indicate a higher incidence of foetal loss and reduced mean litter weight in treated animals.

Table 6.6.2.007-4: Teratological investigation

Glyphosate (mg/kg bw/day)				
Parameter	**0 (control)**	**22**	**103**	**544**
No. of mated females	14	15	14	16
Mean No. of implantations/dam	7.64	8.40	7.07	8.00
Mean No. of dead foetuses/dam	0.07	0.07	0.43	0.56
Foetal loss (%)	0.93	0.79	6.06	7.03
Mean No. of live foetuses/dam	7.57	8.33	6.64	7.44
Mean litter weight (g)	273	283	240	254
Mean foetal weight (g)	36.51	34.33	36.47	34.4
Externally abnormal foetuses	0	0	0	0

Assessment and conclusion by applicant:
The NOAEL for maternal as well as development toxicity was considered to be 544 mg/kg bw/day based on no evidence of maternal or developmental toxicity attributable to glyphosate in either the pre- or postnatal groups.

Assessment and conclusion by RMS:
The conclusion above refers to the part of the study investigating effects on the rat. The part of the study investigating rabbit was not included by the applicant. Since the study was not performed according to GLP and any guideline, several parameters are lacking and data is not clearly and completely presented. Consequently, a robust assessment of data cannot be made and NOAEL/LOAELs can thus not be set. Therefore, in contrast to the previous assessment when the study was considered supplementary, the RMS does not consider the study acceptable.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. Sex ratio was not calculated. Uterine weight was not recorded.

B.6.6.2/08

Data point	EU data requirement No. CA 5.6.2/008
Report author	[redacted] et al.
Report year	1980
Report title	Teratology study in rats
Report No.	401-054
Document No.	M-644179-02-1
Guidelines followed in study	According to the applicant, the study was performed before adoption of OECD Guideline, but in general accordance with OECD Guideline 414 (1981)
Deviations from current test guideline	Stated by the applicant: The following endpoints were not assessed: food consumption, weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant, the deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414. Additional deviations noted by the RMS: • Food consumption was not recorded • Tabulated data on clinical observations are not included, only described in text • uterine weight was not recorded • fetal body weight by sex
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	No. The study report includes a signed quality assurance statement.
Acceptability/Reliability	Acceptable

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: XHJ-64
Purity/Radiochemical purity: 98.7%

Vehicle: 0.5 % aqueous Methocel®

Groups of 25 mated female Charles River COBS® CD® rats received doses of 0, 300, 1000 and 3500 mg Glyphosate/kg bw/day by gavage from day 6 to day 19 of gestation. The day that mating was detected (presence of a copulatory plug or sperm was designated day 0.

Females were observed daily for mortality and overt changes in appearance and behaviour prior to treatment and daily for mortality and clinical signs on days 6 through 20 of gestation. Dams not surviving to the scheduled sacrifice were necropsied to find the cause of death. Individual maternal body weights were recorded on gestation days 0, 6, 9, 12, 16 and 20 but food consumption was not recorded.

All surviving females were sacrificed on gestation day 20 whereupon the uterus was excised and weighed and the foetuses removed. The location of viable and nonviable foetuses, early and late resorptions and the number of total implantations and corpora lutea were recorded. The abdominal and thoracic cavities and organs of the dams were examined for grossly evident morphological changes. All foetuses were individually weighed and examined for external malformations and variations, including the palate and eyes. Each foetus was externally sexed and individually identified. Approximately one-half of the foetuses were examined for visceral changes and the remaining one-half of the foetuses were fixed and stained for skeletal examination. The sensitivity of the rat strain was demonstrated in a separate study with Vitamin A.

RESULTS

Six rats in the high-dose group (i.e. 3500 mg/kg bw/day) died, one each on gestation days 10 and 17 and two each on gestation days 11 and 12. The study report contains no record of clinical signs but according to the study author the clinical signs noted were more or less restricted to high dose animals with diarrhoea occurring primarily either prior to death or during the last days of treatment. Breathing rattles and inactivity were noted only in rats at 3500 mg/kg bw/day and red matter in the region of the nose, mouth, forelimbs or dorsal head was noted prior to death. The inactivity stated to be observed in all rats at 3500 mg/kg bw/day each day, beginning mid-way through the treatment period, approximately l/2 to 6 hours after dosing. Inactivity was not present at subsequent dosing until the last few days of treatment when animals were inactive before and after dosing. Clinical signs in the low and mid dose groups were stated to be similar to controls.

There were no significant differences in mean maternal body weight gain in low and mid dose groups however, a definite reduced mean maternal body weight gain over the treatment period was noted at 3500 mg/kg bw/day. This mainly due to a mean maternal body weight loss during the first three days of treatment.

Table 6.6.2/008-1: Mean body weights (g ± SD) and body weight gain (g)

Glyphosate technical (mg/kg bw/day)				
Parameter	**0 (control)**	**300**	**1000**	**3500**
Mated females	25	25	25	25
No of animals included in assessment (pregnant animals)	22	20	21	17*
Body weights (g)				
Day 0	270 ± 14.4	270 ± 22.8	274 ± 15.9	261 ± 16.8
Day 6	297 ± 16.9	295 ± 22.9	302 ± 18.5	288 ± 15.8
Day 9	305 ± 17.7	303 ± 23.7	307 ± 16.5	275 ± 28.4
Day 12	318 ± 19.3	316 ± 26.2	322 ± 17.7	299 ± 31.4
Day 16	350 ± 19.1	346 ± 32.6	352 ± 21.3	326 ± 26.3
Day 20	416 ± 23.1	403 ± 47.0	416 ± 22.1	373 ± 43.1
Corrected body weight (corrected for uterus weight)	336 ± 19.6	334 ± 27.9	335 ± 17.6	311 ± 32.9 (↓7%)
Body weight gain (g)				
Days 0 - 6	27	25	28	27

Day 6 - 9	8	8	5	-13
Day 9 - 12	13	13	15	24 (↑85%)
Day 12 - 16	32	30	30	27 (↓16%)
Day 16 - 20	66	57	64	47 (↓29%)
Day 0 - 20	146	133	142	112 (↓23%)

* Initial group size of pregnant animals was n=23, further reduced in the course of the study due to 6 mortalities

The mean number of viable foetuses, late or early resorptions, post-implantation loss, corpora lutea, the foetal sex distribution and mean foetal body weight were comparable between low and mid-dose animals and controls. A statistically significant decrease in the mean number of total implantations and viable foetuses was noted in low dose group but in the absence of similar effects at higher doses it was considered a random finding. In high dose animals statistically significant decreases in total implantations and in the number of viable fetuses/dam was noted. The post-implantation loss was increased in high dose animals although statistical significance was not achieved. Since ovulation and implantation occurred prior to treatment, the decreases in total implantation and the slight decrease in corpora lutea are not considered treatment related.

At 3500 mg/kg bw/day, mean foetal weights were reduced in comparison with controls with differences from control values attaining statistical significance. At 300 and 1000 mg/kg bw/day mean foetal weights were similar to those of the control group. No differences in foetal sex ratios were noted between test item-treated groups and the control group.

Table 6.6.2/008-2: Litter data

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
Animals assigned (mated)	25	25	25	25
Non-pregnant animals	3	5	4	2
Dams with pregnancy status not determined (intercurrent death)	0	0	0	6
Dams with total resorption	0	0	0	1
Animals with live young on Day 20	22	20	21	16
Mean no. of corpora lutea ± S.D.	15.9 ± 1.67	15.2 ± 3.30	16.1 ±1.81	14.8 ± 1.64
Mean no. of implantations ± S.D.	15.0 ± 1.11	12.1 ± 4.45**	14.8 ± 2.21	12.8 ±3.77* (↓15%)
Mean post-implantation loss ± S.D.	0.6 ± 090	0.2 ± 0.52	0.5 ± 0.81	1.2 ± 1.25
Mean no. of live foetuses/litter ± S.D.	14.4 ± 1.26	11.9 ± 4.36*	14.3 ± 2.08	11.5 ± 4.12* (↓20%)
Mean Foetal Weight (g ± S.D.)	3.5 ± 0.21	3.7 ± 0.66	3.6 ± 0.19	3.2 ± 0.34** (↓9%)
Sex Ratio (% male foetuses)	50.3	50.2	56.0	49.5

* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$

The number of skeletal and visceral malformations and the number of skeletal variations was higher in high-dose animals compared to controls. Several foetuses of high dose animals with either a malformation classified as

dwarfism or bent tails were found in single litters3. These two increases were considered genetic in origin by the study author since bent tail and dwarfism occurred in several foetuses in a single litter in the historical control data. An increase in the number of litters and foetuses with unossified sternebrae was noted in the 3500 mg/kg bw/day dosage group and was considered a developmental variation.

Table 6.6.2/008-3: Summary of foetal malformations and developmental and genetic variations

Glyphosate technical (mg/kg bw/day)				
Observation	**0 (control)**	**300**	**1000**	**3500**
No. of litters examined	22	20	21	16
No. of foetuses examined externally	316	237	300	196
No. of foetuses examined viscerally	155	119	150	97
No. of foetuses examined skeletally	161	118	150	99
Total % foetuses with malformations (% affected litters)	0.9 (13.9)	0 (0)	0 (0)	5.1 (16.8)
Number of foetuses with skeletal malformations litters)	1(1)			9(2)
% foetuses with skeletal malformations (% affected litters)	0.3 (4.5)	0 (0)	0 (0)	4.6 (12.5)
Number of foetuses with visceral malformations (litters)	2 (2)	0	0	7(2)
% foetuses with visceral malformations (affected litters)	0.6 (9.1)	0 (0)	0 (0)	3.6 (12.5)
Malformations % affected foetuses (% affected litters)				
1 mm vesicle over posterior fontanelle	0 (0)	0 (0)	0 (0)	0.5 (6.3)
Brain anomaly	0.3 (4.5)	0 (0)	0 (0)	0 (0)
Dwarfism	0 (0)	0 (0)	0 (0)	1.5 (6.3)
Rib forked	0.3 (4.5)	0 (0)	0 (0)	0 (0)
Tail threadlike, no anus	0.3 (4.5)	0 (0)	0 (0)	0 (0)
Tail bent	0 (0)	0 (0)	0 (0)	3.1 (6.3)
Developmental variations % affected foetuses (% affected litters)				
27 presacral vertebrae	0 (0)	0.8 (5.3)	0 (0)	0 (0)
14th rudimentary rib(s)	11.2 (40.9)	16.1 (42.1)	16.7 (66.7)	13.1 (50.0)
7th cervical rib	0.6 (4.5)	0.8 (5.3)	0 (0)	0 (0)
Hyoid unossified	1.2 (9.1)	0 (0)	2.7 (14.3)	0 (0)
Reduced	0.6 (4.5)	1.7 (10.5)	0.7 (4.8)	0 (0)

3 Dwarfism was observed in 3 foetuses (1 litter) and bent tail in 6 foetuses (1litter). According to the historical control data included, dwarfism and bent tail were each observed in 5 foetuses (1 litter) in 6955 foetuses from 524 litters.

ossification of skull				
Sternebrae #5 and/or #6 unossified	8.1 (36.4)	5.9 (26.3)	11.3 (38.1)	28.3 (68.8)
Other sternebrae unossified	0.6 (4.5)	0 (0)	0 (0)	6.1 (18.8)
Retrooesophgeal right subclavian	0 (0)	0 (0)	0 (0)	1.0 (6.3)
Renal papilla not developed nad/or distended ureter	1.9 (13.6)	0.8 (5.0)	2.7 (14.3)	4.1 (25.0)

Assessment and conclusion by applicant:
This study from 1980 is considered valid in spite of deviations to the recent OECD guideline (OECD 414, 2018).

The maternal NOAEL was set at 1000 mg/kg bw/day based on mortality, clinical signs and decreased body weight gain at 3500 mg/kg bw/day. Foetal effects were also confined to the top dose level of 3500 mg/kg bw/day. Foetal weights were significantly reduced. The mean number of viable foetuses per litter and the mean foetal weight were decreased. There was a significant increase in early resorptions causing a slight increase in total post-implantation loss.The total number of foetuses with malformations was increased at the highest dose level but the number of affected litters was identical to that in the control groups. Since the incidence and type of malformations were similar to those from historical control data, it was concluded that these findings were not related to treatment. The higher number of foetuses with unossified sternebrae in this dose group was considered an adverse effect of compound administration secondary to maternal toxicity. However, this a developmental variation not a malformation. Thus, the NOAEL for the maternal as well as for foetotoxicity was 1000 mg/kg bw/day in this study.

Assessment and conclusion by RMS:
The RMS agrees with a NOAEL for maternal as well as for foetotoxic effects at 1000 mg/kg bw/day. The effects noted, i.e. maternal deaths, reduced body weight gain, increase in early resorptions, decreases in mean fetal body weight, in mean number of viable fetuses and an increase in visceral and skeletal malformations/variations were only observed at a dose of 3500 mg/kg bw/d which is a dose far above the limit dose and with a maternal mortality of 25%. The previous evaluation is thus confirmed.

ED related parameters not investigated: Weight and histopathological changes of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses. Blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. Uterine weight was not recorded.

B.6.6.2.2. Rabbit

B.6.6.2/09

Data point	EU data requirement No. CA 5.6.2/009
Report author	[redacted]
Report year	1996
Report title	Glyphosate acid: Developmental toxicity study in the rabbit
Report No.	[redacted]/P/5009
Document No.	Not reported
Guidelines followed in study	OECD 414 (1981), EEC B.31 (1988),US-EPA 83-3
Deviations from current test guideline	Stated by the applicant: Dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy.

	Additional deviations noted by the RMS: • Individual data not available • Fetal body weight by sex was not reported • weight and histopathological changes of the thyroid glands of the dams • anogenital distance (AGD) in foetuses, • indication of incomplete testicular descent/cryptorchidism in male foetuses, • blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. According to the applicant, the deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Acceptable

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: P24
Purity/Radiochemical purity: 95.6%
Vehicle: Deionised water

Doses of 0, 100, 175 or 300 mg glyphosate/kg bw/day was administered groups of 20 time-mated New Zealand White female rabbits by gavage from gestation day 8-20. The day of mating was considered day 1. The dose levels are claimed to be selected based on results of a preliminary dose finding study (study report not included and results not further described).

Animals were checked for clinical signs of toxicity, moribundly or behavioural changes once daily during the pre- and post-dosing periods and twice daily (before and after dosing) during the dosing period. Body weights were recorded on arrival, on day 4, prior to dosing on days 8 to 20 and on days 23, 26 and 30 of gestation and the food consumption was recorded on days 4-8, days 8-11, days 11-14, days 14-17, days 17-20, days 20-23, days 23-26 and days 26-30 of gestation. All rabbits were terminated on day 30 and subjected to post-mortem examinations including an external observation and an examination of the thoracic and abdominal viscera. Where there was no clear evidence of implantation, the uterus was removed and stained to determine whether or not implantation had occurred. For pregnant animals the intact gravid uterus (minus ovaries and trimmed free of connective tissue) was removed and weighed and ovaries, uterus and contents where examined. Number of corpora lutea, number and position of implantations, number of live foetuses, foetus weight and early and late intrauterine deaths were determined for each dam.

Each foetus was examined externally together with an examination of the oral cavity. All foetuses were then examined internally for visceral abnormalities, sexed, eviscerated and fixed. following approximately 24 h the head of each foetus was cut along the fronto-parietal suture line and the brain was examined for macroscopic abnormalities. The carcasses were then further processed, stained and the remaining stained foetal skeletons were examined for abnormalities and assessed for the degree of ossification. The skeletal ossification of the manus and pes was assessed using a scale from 1 (good) to 6 (poor).

RESULTS

Intercurrent deaths occurred in all groups, including controls, one in the control and two in each of the 100, 175 and 300 mg/kg/ bw/day groups. One dam in the control group showed weight loss, reduced food consumption, signs of diarrhoea, mucus in the faeces, few faeces and staining in the genital area during the post-dosing period

and aborted on day 30. The post-mortem examination showed changes in the stomach and caecum. Similarly, the dam in the low dose group showed slight body weight loss and reduced food consumption between days 4 and 8 (i.e. prior to the onset of dosing) and onwards until the animal aborted its litter on day 19. The post-mortem examination showed a mass in the right inguinal region of the abdominal cavity. A second animal in this group aborted its litter on day 25 also following a weight loss and reduced food consumption from day 14. The mid-dose dam was sacrificed for humane reasons on day 23 following body weight loss and reduced food consumption from day 4 and onwards. By day 23, the animal was thin and subdued and all uterine implantations were found dead. A second animal in this group aborted its litter on day 22 following slight weight loss from day 14 and reduced food consumption from day 4. The two high-dose animals aborted their litters on days 24 and 23, respectively. Both animals showed a reduction in food consumption from day 11 and body weight loss from day 11/13. The post-mortem examination revealed a hair-like substance in the stomachs of both animals.

The clinical signs observed in remaining animals included an increased incidence of animals producing few faeces, with signs of diarrhoea or with staining in the genital area in high dose animals. The production of few faeces and signs of diarrhoea was also increased in mid dose dams. There were no clinical effects observed in low dose dams.

Table 6.6.2.009-1: Observed clinical signs during the dosing period

	Number of rabbits affected in dose group			
	Control	**Low**	**Intermediate**	**High**
Clinical sign	**(0 mg/kg be/day)**	**(100 mg/kg bw/day)**	**(175 mg/kg bw/day)**	**(300 mg/kg bw/day)**
Blood on tray	0	2	2	1
Cold	0	0	1	0
Dry sores 1 or more areas	0	1	0	0
Ears torn	0	2	1	1
Eye opaque	0	1	0	0
Few faeces on tray	3	3	9	9
Mucus in faeces	1	0	0	0
No faeces on tray	0	1	2	3
Scabs in 1 or more areas	4	6	3	3
Signs of diarrhoea	4	5	11	19
Staining in genital area	2	2	3	11
Subdued behaviour	0	0	1	0
Thin	0	0	1	2
Urine coloured	0	1	1	0
Wet sores in 1 or more areas	2	0	1	0

The maternal body weight was reduced in high dose animals but all except one showed signs of recovery during the post-dosing period. The reduced bodyweight gain correlated with a statistically significantly reduced food consumption during the dosing period. The bodyweight gain in high dose dams was 32% lower than controls whereas the bodyweight gain in low and mid dose groups was higher than in controls. Since both the uterine weight and the mean foetal weights were lower in high dose animals compared to controls, this seems to be an intra-uterine effect (Table 6.6.2.009-4).

Table 6.6.2.009-2: Mean body weight development and bodyweight gain (in g) during gestation

	0 (Control)	**100**	**175**	**300**
Animals per group	17	18	17	17
Day of gestation 8	3924	3771	3822	3815
9	3845	3837	3834	3823
10	3857	3863	3856	3830
11	3885	3873	3874	3854
12	3894	3879	3877	3856

13	3917	3905	3902	3880
14	3942	3932	3930	3875
15	3975	3982	3939	3896
16	4020	4031	3959	3907* (↓3%)
17	4049	4053	3982	3923* (↓3%)
18	4063	4051	3990	3914** (↓4%)
19	4085	4061	4005	3927** (↓4%)
20	4088	4059	3995	3926** (↓4%)
23	4177	4118	4049*	3951** (↓5%)
26	4236	4210	4169	4093* (↓3%)
30	4313	4294	4256	4183
Day 8-20	164	288 (↑76%)	173 (↑55%)	111 (↓32%)

* Significantly different from control at p<0.05
** Significantly different from control at p<0.01

Table 6.6.2.009-3: Mean food consumption (in g/rabbit/day) during gestation

Dose level in mg/kg bw/day				
Day of gestation	**0 (Control)**	**100**	**175**	**300**
4 - 8	155	144	139	160
During Dosing 8 - 11	183	171	158*	149** (↓19%)
11 - 14	172	162	137** (↓20%)	131** (↓24%)
14 - 17	172	163	123** (↓28%)	98** (↓43%)
17 - 20	177	168	143* (↓19%)	115** (↓35%)
Post Dosing 20 - 23	185	176	180 (↓3%)	172 (↓7%)
23 - 26	158	160	179 (↑13%)	179 (↑13%)
26 - 30	137	148	166* (↑21%)	175* (↑28%)

* Significantly different from control at p<0.05
** Significantly different from control at p<0.01

The macroscopic examination did not show any treatment-related changes in any of the investigated tissues, including the ovary and uterus. There were no differences in the number of pregnant dams or other litter parameters except for a non-statistically significant increase of the intrauterine deaths and a statistically significant reduction of foetal weight. The proportion of male foetuses was statistically significantly increased in the mid- dose group however in the absence of a dose-related trend, this finding was considered incidental. There was no adverse effect on the number or survival of the foetuses. There was a statistically significant reduction in mean foetal weight in the high dose level group, in comparison with the control group. According to the study author this difference was attributable to two litters from the dams which had the most severe reductions in bodyweight with a particularly low mean pup weight (29.6 g and 20.3 g respectively). However, in the absence of individual data, this cannot be verified.

Table 6.6.2.009-4: Intergroup comparison of litter data

Glyphosate acid (mg/kg bw/day)				
Observation	**0 (control)**	**100**	**175**	**300**
# Animals Pregnant	18	20	19	19
# Intercurrent deaths	1	2	2	2
Gravid Uterine Weight (g) (mean ± S.D.)	580 ± 117	520 ± 121	493 ± 110	505 ± 119
Mean Placental Weight (mg)				
No. of corpora lutea (mean ± S.D.)	10.8 ± 2.2	11.0 ± 1.6	11.1 ± 1.3	11.2 ± 1.4
No. of implantations (mean ± S.D.)	9.7 ± 2.1	9.0 ± 1.8	9.1 ± 2.5	9.8 ± 1.9
No. of Live Foetuses (mean ± S.D.)	8.4 ± 1.8	8.2 ± 2.2	7.9 ± 2.2	8.5 ± 2.3
Implanation Loss (% mean ± S.D.) Pre	10.7 ± 11.0	18.2 ± 11.1	18.1 ± 20.8	12.8 ± 11.9

Post	11.7 ± 12.0	9.5 ± 16.7	12.1 ± 9.7	13.6 ± 16.6
Intra Uterine Deaths (% mean ± S.D.) Early	6.2 ± 9.7	7.5 ± 17.0	8.1 ± 8.1	11.0 ± 16.0
Late	5.5 ± 10.4	1.9 ± 4.5	4.0 ± 4.9	2.5 ± 8.3
Mean Litter Weight (g) ± S.D.	371 ± 75	350 ± 88	333 ± 79	337 ± 91
Mean Foetal Weight (g) ± S.D.	44.4 ± 4.3	43.3 ± 3.9	43.2 ± 5.7	40.7* ± 7.8 (↓8%)
Sex Ratio	0.41	0.46	0.58**	0.45

* Significantly different from control at $p<0.05$
** Significantly different from control at $p<0.01$

The number of foetuses with major defects was 3/143 (2/17 litters), 1/147 (1/18 litters), 0/135 (0/17 litters) and 2/144 (2/17 litters) in the control, 100, 175 and 300 mg/kg bw/day groups, respectively. The proportion of foetuses with minor external visceral defects was similar for all groups, including the control and there was no significant difference in litter incidences for minor external/visceral defects. Consideration of the specific defects provided no evidence for an adverse effect of glyphosate acid (see tables below).

Table 6.6.2.009-5: Summary of the type and incidence of major defects

	Control	**Low (100 mg/kg bw/day)**	**Intermediate (175 mg/kg bw/day)**	**High (300 mg/kg bw/day)**
Major foetal defects				
Heart single ventricle, ventricle walls thickened, aorta enlarged, pulmonary artery reduced	0/143	1/147	0/135	1/144
Encephalocoele (gross malformation of the skull)	0/143	0/147	0/135	1/144
Cebocephaly, internal hydrocephaly, maxillae fused and shortened, aorta enlarged, persistent truncus arteriosus	1/143	0/147	0/135	0/144
Shortened upper and lower jaws, cleft lip, cleft palate, nares absent, forepaws flexed (right extremely, left slight)	1/143	0/147	0/135	0/144
Reduced number of lumbar vertebrae (25 pre-sacral vertebrae)	1/143	0/147	0/135	0/144

Table 6.6.2.009-6: Summary of the type and incidence of major defects (litter incidences)

Major foetal defects	Control	Low	Intermediate	High
	(0 mg/kg bw/day)	(100 mg/kg bw/day)	(175 mg/kg bw/day)	(300 mg/kg bw/day)
Number of litters affected in dose group*				
Heart single ventricle	0/17	1/18	0/17	1/18
aorta enlarged	1/17	1/18	0/17	1/18
pulmonary artery reduced	0/17	1/18	0/17	1/18
Encephalocoele (gross malformation of the skull)	0/17	0/18	0/17	1/18
Cebocephaly, internal hydrocephaly, maxillae fused and shortened, Shortened upper and lower jaws, cleft lip, cleft palate, nares absent	1/17	0/18	0/17	0/18
persistent truncus arteriosus	1/17	0/18	0/17	0/18
forepaws flexed (right extremely, left slight)	1/17	0/18	0/17	0/18
Reduced number of lumbar vertebrae, 25 pre-sacral vertebrae	1/17	0/18	0/17	0/18

* number affected / total number

The proportion of foetuses with minor external visceral defects was similar for all groups, including the control but the proportion of foetuses with minor skeletal defects was statistically significantly increased in the low and high dose groups but not in the 175 mg/kg bw/day group. The proportions of foetuses (litters) affected were 58/143 (16/17), 82/147 (18/18), 59/135 (16/17) and 79/144 (17/17) in controls, low, mid and high dose groups, respectively. The specific defects noted included a statistically significant increased incidence of foetuses in the high dose group with partially ossified transverse processes on the 7th cervical vertebra (8 foetuses in 2 litters compared to 1 foetus in 1 litter in controls), unossified transverse processes on the 7th lumbar vertebra (14 foetuses in 4 litters compared to 4 foetuses in 3 litters in controls) or partially ossified 6th sternebra (16 foetuses in 7 litters compared to 4 foetuses in 2 litters in controls), see table 6.6.2.009-8. None of the specific minor defects were statistically significantly increased in the low or intermediate dose groups.

None of the foetuses were found to have an external/visceral variant. The proportion of foetuses with skeletal variants was statistically significantly increased in the high dose group due to a slight, but not statistically significant, increase in the incidence of foetuses with partially ossified odontoids (62 foetuses in 15 litters) or with 27 pre-sacral vertebrae (37 foetuses in 12 litters). The slightly higher mean manus score observed in the high dose group, in comparison with the control group, was due to a slight reduction in ossification as shown by the increase in incidence of foetuses scoring 4 or 5. A similar response was apparent from the pes scores. According to the study author, the slight reduction in ossification was influenced by the low weight foetuses. This seems likely however in the absence of individual data this cannot be verified.

Table 6.6.2.009-7: Incidence of foetal malformations, minor defects and variations in rabbits treated with glyphosate acid

Dose level (mg/kg bw/day)				
Foetal findings	**0**	**100**	**175**	**300**
No. of litters examined	17	18	17	17
No. of foetuses examined	143	147	135	144
Skeletal malformations				
Total no. of foetuses with major defects	3	0	0	1
Total no. of litters with major defects	2	0	0	1
Percentage of litters with major defects (%)	11.8	0.0	0.0	5.9
Skeletal minor defects**				
Total no. of foetuses with minor defects	58	82*	59	79*
Total no. of litters with minor defects	16	18	16	17
Percentage of litters with minor defects (%)	94.1	100	94.1	100
Skeletal variations				
Total no. of foetuses affected	119	129	116	132*
Total no. of litters affected	17	18	17	17
Percentage of litters affected (%)	100	100	100	100
External and visceral malformations				
No. of foetuses with major defects	2	1	0	2
No of litters with foetuses with major defects	2	1	0	2
Percentage of litters with foetuses with major defects (%)	11.8	5.6	0.0	11.8
External and visceral - minor defects				
No. of foetuses with minor defects	12	7	9	11
No of litters with foetuses with minor defects	8	5	8	7
Percentage of litters with foetuses with minor defects (%)	47.1	27.8	47.1	41.2

* Statistically significant from control ($p<0.05$)

** Small, generally transient deviations which are considered not to be incompatible with survival and which frequently represent a manifestation of delayed development e.g. reduced ossification. The minor classification is used for observations which generally occur at low frequency, in less than 10% ofthe control population. The variant classification is used for

observations which consistently occur at a frequency greater than 10%. Temporal shifts are known to occur • in the frequency of some observations and this is also taken into account at the time of classification.

Table 6.6.2.009-8: Incidence of minor defects in rabbits treated with glyphosate acid

	Control	Low (100 mg/kg bw/day)	Intermediate (175 mg/kg bw/day)	High (300 mg/kg bw/day)
	Foetal/litter incidence in number (percentage)			
Partially ossified transverse processes on the 7th cervical vertebra, ,	1 (0.7%) / 1 (5.9%)	2 (1.4%) / 1 (5.6%)	3 (2.2%) /2 (11.8%)	8*(5.6%) / 2 (11.8%)
Unossified transverse processes on the 7th lumbar vertebra,	4 (2.8%) / 3 (17.6%)	1 (0.7%) / 1 (5.6%)	3 (2.2%) / 3(17.6%)	14* (9. 7%)/ 4 (23.5%)
Partially ossified 6th sternebra,	4 (2. 8%)/ 2 (11.8%)	9 (6.1%)/ 6 (33.3%)	9 (6.7%)/ 5 (29. 4%)	16** (11.1%)/ 7 (41.2%)
27 pre-sacral vertebrae	23 (16.1%) / 10 (58.8%)	29 (19.7%)/ 12 (66.7%)	26 (19.3%)/ 9 (52. 9%)	37 (25.7%)/ 12 (70. 6%)
Partially ossified odontoids (variation)	50(35.0%) 15 (88.2%)	55 (37. 4%) 15 (83.3%)	47 (34.8%) 13 (76.5%)	62 (43 .1%) 15 (88.2%)

6.6.2.009-9: Teratology study in the rabbit ([redacted] 1996): Intergroup comparison of manus/pes assessment

Glyphosate acid (mg/kg bw/day)				
Observation	**0**	**100**	**175**	**300**
Number of Litters	17	18	17	17
MANUS Scores				
Score 1	0	0	0	1
Score 2	25	20	19	19
Score 3	107	113	110	96
Score 4	10	13	6	21
Score 5	1	1	0	7
Mean MANUS Score per litter (± S.D.)	2.88 ± 0.36	2.97 ± 0.27	2.89 ± 0.23	3.05 ± 0.57
PES Scores				
Score 1	134	135	131	116**
Score 2	9	12	4	28**
Mean PES Score per litter (± S.D.)	1.07 ± 0.14	1.08 ± 0.05	1.03 ± 0.05	1.18 ± 0.32

** Significantly different from control at $p < 0.01$

Assessment and conclusion by applicant:

The study is considered valid, though treatment duration covered the period from implantation through organogenesis rather than through to the day prior to necropsy, as recommended in the current OECD 414 guideline.

The oral administration of glyphosate acid to time-mated rabbits by gavage at a maximum dose level of 300 mg/kg bw/day from Gestation Day 8-20 resulted in maternal toxicity at 175 and 300 mg/kg bw/day. There were no treatment-related effects on pregnancy or foetuses at any dose level that could not be attributed to maternal toxicity.

Therefore the NOAEL was considered to be 100 mg/kg bw/day for maternal toxicity. The NOAEL for developmental toxicity was considered to be 175 mg/kg bw/day.

Assessment and conclusion by RMS:
Treatment resulted in diarrhoea and reduced food consumption in mid and high dose animals and reduced body weight gain in high-dose dams, possible an intra-uterine effect since the uterus weight was reduced as well as the mean foteal weights. The bodyweights of foetuses from high dose dams were reduced by 8% and a reduced ossification was observed. The visceral and skeletal defects noted did not show a clear dose-response and since similar effects generally occurred at comparable frequencies also in the control group, they are not considered related to treatment.

The RMS agrees with the maternal NOAEL of 100 mg/kg bw/d based on clinical signs, reduced food consumption and reduced bodyweight gain and a developmental NOAEL of 175 mg/kg bw/d based on reduced foetal weights. Teratogenic effects were not observed.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH).

B.6.6.2/10

Data point	EU data requirement No. CA 5.6.2/010
Report author	[redacted]
Report year	1996
Report title	Glyphosate technical: Oral gavage teratology study in the rabbit
Report No.	434/020
Document No.	Not reported
Guidelines followed in study	OECD 414 (1981), JMAFF 59 NohSan 4200 (1985), US-EPA 83-3 (1984)
Deviations from current test guideline	Stated by the applicant: Dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy. Additional deviations noted by the RMS: • Individual foetal data not available • Fetal body weight by sex was not reported • weight and histopathological changes of the thyroid glands of the dams, • anogenital distance (AGD) in foetuses, • indication of incomplete testicular descent/cryptorchidism in male foetuses, • blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Acceptable

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: H95D161A
Purity/Radiochemical 95.3%

purity:
Vehicle: 1 % carboxymethyl cellulose

Preliminary study (study number 434/019)
Groups of six mated New Zealand white female rabbits received 0, 50, 200 or 400 mg/kg bw/day test substance in 1 % carboxymethyl cellulose by gavage from gestation day 7-19 (the day of copulation was designated day 0 of gestation). The dose levels were chosen based on results of a preliminary dose finding study with 6 nulliparous rabbits receiving doses of 500 or 1000 mg/kg bw that resulted in toxicity (scours, fluid filled caecum, stomach ulceration, body weight loss, reduced food consumption) indicating that dose levels at and above 500 mg/kg bw were considered too high for a prolonged study.

Main study
Based on the results from the preliminary study, doses of 0, 50, 200 or 400 mg/kg bw/day test substance in 1% carboxymethyl cellulose were administered groups of 18 mated New Zealand white female rabbits by gavage from gestation day 7-19. Animals were checked for clinical signs of toxicity, ill-health or behavioural changes twice daily (before and after dosing) during the dosing period, individual body weights were recorded on day 3, 7, 10, 13, 16, 19, 22, 25 and 29 of gestation and food consumption was recorded on days 3 to 7, days 7 to 10, days 10-13, days 13-16, days 16-19, days 19-22, days 22-25 and days 25-29 of gestation.

Females were sacrificed on day 29 of gestation, examined for macroscopic abnormalities and subjected to caesarean sectioning. The ovaries and uteri were removed, weighed and examined to determine the number of corpora lutea, the number and position of implants and the number of dead or live foetuses. Resorptions and foetal deaths were classified into implantation sites, placental remnants, and macerated foetuses according to the difference in developmental stage at which deaths had occurred. Foetal sex, external and internal foetal appearance and foetal weight was determined. After examination of the ovaries and conceptuses, each female was necropsied.

All foetuses were dissected and examined for visceral abnormalities macroscopically. The heads of alternate foetuses were removed, fixed and following a minimum of 14 days, examined for visceral anomalies under a low power binocular microscope. The foetuses were eviscerated, processed and stained to examine skeletal development and anomalies.

RESULTS

PRELIMINARY STUDY
Two high-dose dams were killed in extremis on Days 22 and 11 respectively post coitum. One of these was killed as it had aborted foetuses. The pathological examination revealed caecum, appendix and colon filled with dark fluid, pale kidneys, accentuated lobular pattern of liver and blood around the vagina. The other high-dose dam was killed as it was bleeding from the vagina. Similarly, caecum, appendix and colon were filled with dark fluid in this animal and rectum was filled with soft contents, digested blood and brown staining around the anus. Common clinical signs observed in the high dose group included scours and reduced faecal output (more prevalent than in control and lower dose levels) and isolated clinical observations of lethargy and ptosis were noted. Blood was noted on the tray of one mid-dose animal on one day. The body weight gain in high and mid dose animals was significantly decreased from Day 13 to 19 post coitum and the food consumption was reduced in high dose animals. Both food consumption and bodyweight gain recovered after treatment. Besides the animals killed in extremis there were no effects observed at necropsy, no effects on implantation or litter parameters in treated animals and all foetuses appeared normal. Based on these results, the dose levels 50, 200 and 400 mg/kg were chosen for the main study.

MAIN STUDY
Two high dose rabbits were found dead or moribund; one found dead prior to dosing on day 19 of treatment and the other killed in extremis on day 20 of treatment. Clinical observations by this time included hunched posture, lethargy, ptosis, hypothermia and blood on the litter tray. One mid-dose female was found dead after dosing on Day 16 of treatment but findings at necropsy findings indicated that this was due to mal-dosing.

Clinical signs in remaining animals were similar to those observed in the preliminary study, particularly scours, reduced faecal output and diarrhoea at the high dose level (400 mg/kg bw/day).Vaginal bleeding and blood on tray were noted for one mid-dose dam. Scours were also noted in animals at 200 and 50 mg/kg bw/day as well as

in the control group, but the incidence and duration were not as severe as at the high dose level. There were no clinical signs observed in the non-pregnant dam.

Table 6.6.2.010-1: Observed clinical signs during the dosing period

Number of rabbits affected in dose group#				
Clinical sign	**(0 mg/kg bw/day)**	**Low (50 mg/kg bw/day)**	**Intermediate (200 mg/kg bw/day)**	**High (400 mg/kg bw/day)**
Scours	5/14 (4)	10/18 (0)	7/16 (2)	16/16 (2)
Reduced faecal output	1/14 (4)	1/18 (0)	2/16 (2)	6/16 (2)
Diarrhoea	0/14 (4)	1/18 (0)	0/16 (2)	10/16 (2)
Diuresis	0/14 (4)	0/18 (0)	1/16 (2)	0/16 (2)
Blood on tray	0/14 (4)	0/18 (0)	1/16 (2)	1/16 (2)
Noisy respiration	0/14 (4)	0/18 (0)	1/16 (2)	1/16 (2)
Lethargy	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Ptosis	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Hunched posture	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Hypthermia	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Anal staining	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Subdued behaviour	0/14 (4)	0/18 (0)	0/16 (2)	1/16 (2)
Vaginal bleeding	0/14 (4)	0/18 (0)	1/16 (2)	0/16 (2)

x/y: number affected / total number of animals in group
Figures in parentheses represent the number of animals having no grossly observable conceptus.

In similarity with the results from the preliminary study, the mean body weight gain from days 9 to 29 post coitum was reduced in high-dose dams and the difference was statistically significant ($P<0.05$ to 0.01) from Days 13 to 29 post coitum. Also in the intermediate dose level group a slight reduction (although not statistically significant) was observed from day 9 to day 29 post coitum was noted. It is noted that there was a large variation between animals as indicated by standard deviations (illustrated in table 6.6.2.010-2 by the inclusion of individual values recorded at one of the measurements). In the low dose level group body weight gain was comparable to controls throughout the study period (see table below). This was probably explained by the reduced food consumption during the dosing period which was statistically significant during days 10 to 13 post coitum (26%), days 13-16 (35%) and days 16- to 19 post coitum (38%). The food consumption was comparable to controls during the post-dosing period. At 200 and 50 mg/kg there were no significant differences in food consumption.

Table 6.6.2.010-2: Mean body weight gain during gestation

Body weight change (g) at Day (relative to Day 7)								
Dose level (mg/kg bw)	**No. of animals**	**10**	**13**	**16**	**19**	**22**	**25**	**29**
0 (Control)	14	29	95 SD 135 Ind data (g): 58, 35, 127, -83, -35, 180, 41,15,48, 465,150, 234, 48 (14 animals, 3 non-preg, one found	202	260	314	375	409

			dead)					
50	18	12	75 SD 88.8 (↓21%) Ind data (g): 150, 251, 41,130, -56,126, -16, 58, 57, -69, 186, -76, 58, 53, 129, 108, 136, 90 (18 animals)	158 (↓22%)	223 (↓14%)	278 (↓11%)	325 (↓13%)	395 (↓3%)
200	15	-11	54 SD 62.4 (↓43%) Ind data (g): 88, 117, -18, 60,103,-20, 8, 49, -22, 107, 39, 71, 153, 122,-49 (15 animals, 2 non-preg, one found dead)	143 (↓29%)	198 (↓24%)	263 (↓16%)	309 (↓18%)	294 (↓28%)
400	15	-33	-45* SD 36.7 Ind data (g): 85, 0, 20, -395, -157, -38, -32, -32, -30, 18, -282, 89, -5, 98, 78, -124 (15 animals, 1 non-preg, 1 found dead, 1 killed in extremis)	11** (↓95%)	21** (↓92%)	96** (↓36%)	153** (↓39%)	250* (↓39%)

* Significantly different from control at $p<0.05$.
** Significantly different from control at $p<0.01$.

The post-mortem examinations of the animals that died or was killed in extremis revealed fluid filled large intestines, haemorrhage, ulceration and sloughing of the stomach, duodenum congested and colon, rectum and appendix gas distended indicating a test-substance related effect on the gastrointestinal tract. The animal killed in extremis also had both uterine containing blood and dead foetuses in the uterus, possibly due to maternal toxicity. Other necropsy findings among animals seemed related to mal-dosing.

In the control, low, intermediate and high dose level groups 14, 18, 15, and 15 females, respectively, survived to termination of the main study and were proven to be pregnant. The number and distribution of females that were not pregnant indicate that there were no treatment-related effects on pregnancy rates. Litter size at caesarean necropsy was comparable in all treatment groups.

The litter size was comparable in all treatment groups. There were slight but non-statistically significant increases in late foetal deaths and post implantation loss in the high dose group. This seemed mainly due to one animal that had nine late deaths, resulting in a post implantation loss of 69.2 %. The study author did thus not consider this a treatment-related effect. However, an increase in post implantation loss was also observed in mid dose animals and the increase was statistically significant. The increase was caused by a slight, but not statistically significant, rise in early foetal deaths. Since there was no rise in late foetal deaths the study author did not consider this effect toxicologically significant. Also the applicant states “Since there is no dose response observed, early foetal deaths, late foetal deaths, and total foetal deaths (the sum of early plus late foetal deaths), increases in total foetal deaths and post-implantation loss at 200 mg/kg bw/day were considered not to be treatment-related.” The RMS agrees that there is no apparent dose-response however it is also noted that the number of implantations were higher in high dose animals than in mid-dose animals which may to some extent influence numbers. Therefore, the toxicological significance of the increased incidence of late foetal deaths and post-implantation loss is considered unclear.

Table 6.6.2.010-3: Intergroup comparison of litter data

Glyphosate acid (mg/kg bw/day)				
Observation	**0 (control)**	**50**	**200**	**400**
# Animals Pregnant at Necropsy	14	18	15	15
No. of corpora lutea (mean ± S.D.)	10.9 ± 2.2	10.5 ± 2.4	10.7 ± 2.1	11.5 ± 1.8
No. of implantations (mean ± S.D.)	9.5 ± 2.5	9.1 ± 2.3	8.9 ± 2.5	10.3 ± 2.3
No. of Live Foetuses (mean ± S.D.)	9.1 ± 2.5	8.7 ± 2.4	7.9 ± 2.5	8.9 ± 2.6
Embryonic/Foetal Deaths Early	0.21 ± 0.43	0.22 ± 0.55	0.87 ± 1.06	0.47 ± 0.92
Late	0.14 ± 0.53	0.11 ± 0.32	0.13 ± 0.35	0.93 ± 2.28
Total	0.36 ± 0.63	0.33 ± 0.77	1.00* ± 1.00	1.40 ± 2.35
Implantation Loss (% mean ± S.D.) Pre	12.5 ± 18.2	13.6 ± 9.4	16.4 ± 15.5	9.3 ± 12.5
Post	3.7 ± 6.5	3.6 ± 8.5	11.5* ± 11.4	12.1 ± 18.6
Total Litter Weight (g) ± S.D.	372.0 ± 92.6	334 ± 75.1	321.5 ± 79.2	337.0 ± 84.4
Mean Foetal Weight (g) ± S.D.	41.5 ± 5.5	39.4 ± 5.6	41.7 ± 4.5	38.2 ± 5.2
Sex Ratio	0.56	0.51	0.54	0.51

* Significantly different from control at $p < 0.05$.

At the high dose level, one foetus showed major malformations. This foetus was found to have spina bifida and clubbed and malrotated hind limbs. At the intermediate dose level, two foetuses of two different litters had major malformations. One foetus had retinal infolding and a haemorrhage in the retinal layer, the other acephaly, small kinked tail, bilateral forelimb flexure, interrupted aorta and an intraventricular septal defect. At skeletal examination, this foetus was found to have multiple rib and vertebral column abnormalities. At the low dose

level, three foetuses of two different litters had major abnormalities. In one litter, one foetus had forked ribs with a displaced vertebral centrum. In another litter, one foetus had a small eye with retinal infolding and aphakia. A second foetus from this litter had nostrils close together, and a thin nasal septum not attached at posterior pole near the front of the nasal passages. In the control group, there were two foetuses from two different litters with major abnormalities. One foetus had gastroschisis and the other foetus had an extra vertebral arch resulting in scoliosis (see table 6.6.2.010-4).

These findings were considered to be within the range of normal variation for this species. There were no treatment-related effects on the degree of skeletal development.

Table 6.6.2.010-4: Incidence of foetal malformations and variations in rabbits treated with glyphosate acid

Foetal findings				
Dose level (mg/kg bw/day)	**0**	**50**	**200**	**400**
No. of litters examined	14	18	15	15
No. of foetuses examined	128	157	119	134
Skeletal malformations				
Total no. of foetuses with skeletal malformations	1	0	1	0
Total no. of litters with skeletal malformations	1	0	1	0
Percentage of litters with skeletal malformations (%)	7.1	0.0	6.7	0.0
Skeletal variations				
Total no. of foetuses with skeletal variations	43	48	39	49
Total no. of litters with skeletal variations	13	18	15	15
Percentage of litters with skeletal variations (%)	92.8	100	100	100
External and visceral findings				
No. of litters examined	14	18	15	15
No. of foetuses examined	128	157	119	134
No of litters with anomalous foetuses	2	5	2	3
Percentage of litters with anomalous foetuses (%)	14.3	27.8	13.3	20
No. of litters with major malformations	2	2	2	1
Percentage of litters with malformed foetuses (%)	14.3	11.1	13.3	6.7

Table 6.6.2.010-5: Incidence of foetal external, visceral and skeletal findings discussed in text

Foetal findings number of foetuses in category/ number of litters in category/group mean litter				
Dose level (mg/kg bw/day)	**0**	**50**	**200**	**400**
Foetal external and visceral findings				
Spina bifida	0/0/0%	0/0/0%	0/0/0%	1/1/0.8%
clubbed and malrotated hind limbs	0/0/0%	0/0/0%	0/0/0%	1/1/0.8%
gastroschisis	1/1/0.9%	0/0/0%	0/0/0%	0/0/0%
small kinked tail	0/0/0%	0/0/0%	1/1/0.8%	0/0/0%
Uni/bilateral forelimb flexure	0/0/0%	0/0/0%	1/1/0.8%	1/1/0.7%
interrupted aorta	0/0/0%	0/0/0%	1/1/0.8%	0/0/0%
intraventricular septal defect	0/0/0%	0/0/0%	1/1/0.8%	0/0/0%
retinal infolding	0/0/0%	1/1/1.1%	1/1/1.1%	0/0/0%
haemorrhage in the retinal layer	0/0/0%	0/0/0%	1/1/1.1%	0/0/0%
aphakia	0/0/0%	1/1/0.9%	0/0/0%	0/0/0%
acephaly	0/0/0%	0/0/0%	1/1/0.8%	0/0/0%
nostrils close together	0/0/0%	1/1/0.5%	0/0/0%	0/0/0%
thin nasal septum not attached at posterior pole near the front of the nasal passages	0/0/0%	1/1/0.9%	0/0/0%	0/0/0%
Foetal skeletal development				
multiple rib				
vertebral column abnormalities	0/0/0%	0/0/0%	1/1/0.8%	0/0/0%
one pair of ribs articulate to sarne vertebra • ribs forked	0/0/0%	1/1/0.9%	0/0/0%	0/0/0%
extra vertebral arch resulting in scoliosis.	1/1/1.0%	0/0/0%	0/0/0%	0/0/0%

Assessment and conclusion by applicant:

The study is considered valid, though treatment duration covered the period from implantation through organogenesis rather than through to the day prior to necropsy, as recommended in the current OECD 414 guideline.

The oral administration of glyphosate technical to pregnant rabbits by gavage from gestation Day 7-19 resulted maternal toxicity at 200 and 400 mg/kg bw/day. Therefore the 'No Observed Adverse Effect Level' (NOAEL) was considered to be 50 mg/kg bw/day for maternal toxicity. There were no treatment-related effects on pregnancy or foetuses at any dose level. The NOAEL for developmental toxicity was considered to be 400 mg/kg bw/day.

Assessment and conclusion by RMS:

The RMS agrees with the NOAEL set for maternal toxicity. The toxicological significance of the statistically significant increase in post-implantation loss in mid dose (also increase in high dose) is unclear but at least in

the high dose group it is mainly due to effects in one dam. Moreover, there is no clear dose-response which would have been expected for this type of effect. The incidence of early foetal deaths in the top dose is approximately half the incidence in mid dose and the effect in mid dose thus seems to reflect variation.
Therefore, the RMS agrees also with the developmental NOAEL.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH), uterine weight.

B.6.6.2/11

Data point	CA 5.6.2/011
Report author	[redacted]
Report year	1995
Report title	HR-001: A Teratogenicity Study in Rabbits
Report No.	[redacted] 94-0153
Document No.	M-301383-01-1
Guidelines followed in study	OECD 414 (1981), JMAFF 59 NohSan 4200 (1985), US-EPA 83-3 (1984)
Deviations from current test guideline	Stated by the applicant: Dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy Additional deviations noted by the RMS: • insemination is not considered in OECD TG 414 • weight and histopathological changes of the thyroid glands of the dams, • anogenital distance (AGD) in foetuses, • indication of incomplete testicular descent/cryptorchidism in male foetuses, • blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Acceptable

MATERIALS AND METHODS

Test material:	Glyphosate Technical
Lot/Batch:	T-9 41209
Purity/Radiochemical purity:	97.56%
Vehicle:	Purified water with 0.5% carboxymethylcellulose

Groups of 18 artificially inseminated Japanese White female rabbits received doses of 0, 10, 100 and 300 mg/kg bw/day test substance in carboxymethylcellulose by gavage from Gestation day 6-18. The dose levels were chosen from the results of a preliminary teratogenicity study.

Dams were checked daily for clinical signs of toxicity, ill-health or behavioural changes during the pre- and post-dosing periods and twice daily (before and after dosing) during the dosing period. Individual body weights were recorded on day 0, 6-18, 24 and 27 of gestation and the body weight gains were calculated by subtracting the body weight value on day 0 of gestation from each value determined on days 6 through 27 of gestation. Adjusted weights were calculated by subtracting the gravid uterine weight from the body weight on day 27 of gestation. Food consumption of females was determined on alternate days from day 0 to day 26 of gestation and on days 26 to 27 of gestation. In each interval, daily food consumption (g/rabbit/day) was calculated for each female.

All animals were terminated on day 27 of gestation and the ovaries and uteri were removed, weighed and then examined for the number of corpora lutea and for the number and position of implants and dead or live foetuses. Resorptions and foetal deaths were classified into implantation sites, placental remnants, and macerated foetuses according to the difference in developmental stage at which deaths had occurred. When uterine implants were not grossly apparent, the uteri were stained to detect very early resorptions. After examination of the ovaries and conceptuses, each female was necropsied.

Live foetuses and their placentas were individually weighed and examined for external abnormalities. The eyes were examined for alterations after removing the palpebral skin. The sex of the foetuses was determined by observation of the gonads. Thereafter, each foetus was examined for visceral abnormalities. Then the thoracic and abdominal organs were removed and the remaining skeletons were fixed and examined for skeletal abnormalities.

RESULTS

MORTALITY

One rabbit in the high dose group died on day 20 of gestation without showing any clinical signs. Females with resorptions only had no grossly observable conceptus but had implantation sites in the uteri indicating very early resorptions. These animals were excluded from the statistical evaluation.

Table 6.6.2.011-1: Survival and pregnancy status in maternal rabbits

Dose level (mg/kg bw/day)	No. of females mated	No. of females pregnant	No. of females died or killed	No. of females aborted	No. of females survived	No. of females with resorptions only	No. of females with live foetuses
0	18	18	0	0	18	0	18
10	18	18	0	2	16	1	15
100	18	18	0	0	18	2	16
300	18	18	1	2	15	1	14

CLINICAL SIGNS

Loose stool was observed in four high dose dams and in two high dose dams soiled fur was observed in the perianal region. This was considered due to defecation of loose stool. During the post-dosing period one low-dose dam aborted on day 20 of gestation, and another delivered prematurely on day 27 of gestation. Loose stool remained in two of four high dose dams and one of these two aborted on day 26 of gestation. Although loose stool disappeared from the two other dams, one of these prematurely delivered on day 27 of gestation and the second had hair loss in the dorsal region. Considering that loose stool and subsequent abortion or premature delivery was observed also in the preliminary study (at doses of 300 and 1000 mg/kg bw/d), the observations in the high dose group in the main study is considered related to treatment.

Table 6.6.2.011-2: Observed clinical signs during the dosing period

Number of rabbits affected in dose group#				
Clinical sign	**Control (0 mg/kg bw/day)**	**Low (10 mg/kg bw/day)**	**(Mid 100 mg/kg bw/day)**	**High (300 mg/kg bw/day)**
No abnormalities detected	18/18 (0)	16/17 (1)	15/16 (2)	13/17 (0)
Hair loss	0/18 (0)	1/17 (0)	0/16 (0)	0/17 (0)
Scab on the auricle	0/18 (0)	0/17 (0)	1/16 (0)	0/17 (0)
Soiled fur in the perianal region	0/18 (0)	0/17 (0)	0/16 (0)	2/17 (0)
Loose stool	0/18 (0)	0/17 (0)	0/16 (0)	4/17 (1)*

x/y: number affected / total number of animals in group

* Significantly different from control at $p<0.05$.

Figures in parentheses represent the number of animals having no grossly observable conceptus. These animals were excluded from statistical evaluation.

BODY WEIGHT GAIN

The body weight gain was higher in low and mid dose animals compared to controls during gestation. Also in high dose animals the body weight gain was higher until approximately day 12 after which the body weight gain was lower although differences from controls were not statistically significant. The mean food consumption in the treated groups was comparable to that in the control group throughout the study period.

Table 6.6.2.011-3: Body weight gain (g) during gestation

									Body weight on gestation day							
Dose level	**Days**	**0-6**	**0-7**	**0-8**	**0-9**	**0-10**	**0-11**	**0-12**	**0-13**	**0-14**	**0-15**	**0-16**	**0-17**	**0-18**	**0-24**	**0-27**
0 mg/kg bw/day	Mean	95	113	110	119	122	145	166	183	193	193	192	203	212	260	286
	SD	40	57	65	55	63	64	95	115	132	149	160	165	172	244	278
	No.	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
10 mg/kg bw/day	Mean	104	119	117	135	145	173	195	225	225	234	241	256	250	335	351
	SD	40	48	53	60	64	74	81	84	97	118	126	140	139	186	151
	No.	17	17	17	17	17	17	17	17	17	17	17	17	17	16	15
100 mg/kg bw/day	Mean	129	143	139	156	162	200	213	253	270	280	290	305	309	383	442
	SD	67	74	74	101	90	114	104	109	107	111	111	112	120	160	174
	No.	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
300 mg/kg bw/day	Mean	116	122	121	125	121	151	157	176	175	164 (↓15)	134 (↓30)	145 (↓15)	147 (↓31)	193(↓26)	322 (↑13)
	SD	62	75	87	95	91	115	143	176	200	224	244	256	286	363	216
	No.	17	17	17	17	17	17	17	17	17	17	17	17	17	16	14

PATHOLOGY

The pathological examination of the high-dose dam who aborted revealed yellow-coloured adipose tissue, a hair bolus in the stomach, watery contents in the large intestine and accentuated lobular pattern in the liver. The prematurely delivered rabbit in the high dose group had soiled fur in the perianal region, erosion in the stomach, a hair bolus in the stomach, and watery contents in the caecum. The examination of the rabbit who died showed pale liver and ascites (red) in the abdominal cavity but the cause of death was not established.

The gross pathological findings in surviving animals included hair loss in the lower abdominal or dorsal region in one animal each in the mid and high dose groups; hair bolus in the stomach of one animal each in the control and low dose groups.

PREGNANCY PARAMETERS

Examinations of uterine contents showed no abnormalities and the mean gravid uterine weights and mean numbers of corpora lutea and implants were comparable between the control and the treated groups.

Table 6.6.2.011-4: Intergroup comparison of maternal performance

Glyphosate acid (mg/kg bw/day)				
Observation	**0 (control)**	**10**	**100**	**300**
# Animals mated	18	18	18	18
# Animals Pregnant	18	18	18	18
# Pregnancy status (non-pregnant)	0	0	0	0
# Pregnant does	0	0	0	1

died or killed in extermis				
# Does aborted	0	2	0	2
Does with implantation sites but no observable conceptus **Excluded from calculations**	0	1	2	1
# Litters	18	16	18	15
Gravid Uterine Weight (g) (mean ± S.D.)	407±111	454±127	488±118	416±155
Mean Placental Weight (mg)	4928	5156	4717	4697
No. of corpora lutea (mean ± S.D.)	10.2±2.0	11.7±2.2	12.1±2.0	10.1±2.3
No. of implantations (mean ± S.D.)	8.5±2.8	9.8±2.9	10.4±2.9	8.6±3.3
No. of Live Foetuses (mean ± S.D.)	7.8±2.4	8.7±3.2	9.4±2.7	8.0±3.2
% of Foetal resorption and death	7.1	13.8	8.7	6.5
Males Mean Foetal Weight (g) ± S.D.	35.8±8.1	37.3±5.4	36.7±3.3	36.2±5.4
Females Mean Foetal Weight (g) ± S.D.	35.7±6.7	36.1±5.1	36.0±3.9	34.9±4.4
Sex Ratio	0.49	0.53	0.49	0.47

FOETAL EFFECTS

There were no statistically significant differences in the mean number of live foetuses and mean percent incidences of resorptions and foetal deaths between the control group and the treated groups. It should be noted, however, that females with very early resorptions (does with implantation sites but no observable conceptus, see table 6.6.2.011-4) were excluded from the statistical evaluation, There were no statistically significant differences in sex ratios, mean foetal body weights, mean placental weights, mean number of live foetuses, and mean percent incidences of resorptions and foetal deaths between control and treated groups. However, the number of litters with anomalous foetuses was significantly higher in the high-dose group compared to control group (see table below). This was due to an increase in skeletal malformations, as no external or visceral malformations were noted in foetuses from the high dose group. Since the types of skeletal malformations were inconsistent and there was no clear dose-response in the number of foetuses showing skeletal malformations, the study author considered this a sporadic alteration rather than treatment-related.

Table 6.6.2.011-5: Observed clinical signs during the dosing period

Observations						
Dose level (mg/kg bw/day)	**No. of pregnant females examined**	**No. of females having anomalous foetuses (%)**	**No. of foetuses examined**	**No. of foetuses with external malformations (%)**	**No. of foetuses with visceral malformations(%)**	**No. of foetuses with skeletal malformations (%)**
0	18	1	140	0 (0.0)	0 (0.0)	1 (0.7)
10	15	3	130	0 (0.0)	1 (0.8)	4 (3.1)
100	16	3	150	2 (1.3)	3 (2.0)	6 (4.0)
300	14	5*	112	0 (0.0)	0 (0.0)	5 (4.5)

* Significantly different from control at p<0.05.

The percentage of litters with variations was actually lower in high dose animals compared to controls but the incidence of total no. of litters with skeletal variations was significantly higher in mid dose animals compared to control (see table below) due to a significantly high incidence of lumbar ribs (87.5 % of litters, 27.3 % of the foetuses) compared with the control (72.2 % of litters, 16.4 % of foetuses). The total litter incidence for skeletal variations in the 100 mg/kg bw/day group was 100 %. However, the increased incidence of lumbar ribs in the 100 mg/kg bw/day group was considered a sporadic alteration because the value was within the historical control range (8.1-35.0 % of examined foetuses, data not included), and because no such increase was observed in the 300 mg/kg bw/day group (13.4 %).

Table 6.6.2.011-6: Incidence of foetal malformations and variations

Foetal findings				
Dose level (mg/kg bw/day)	0	10	100	300
Malformations				
No. of litters examined	18	15	16	14
No. of foetuses examined	140	130	150	112
No. of malformed foetuses (external/visceral/skeletal)	0/0/1	0/1/4	2/3/6	0/0/5
Percentage of malformed foetuses (external/visceral/skeletal)	0/0/0.7	0/0.8/3.1	1.3/2.0/4.0	0/ 0/4.5
No of litters with anomalous foetuses	1	3	3	5*
Percentage of litters with malformations (%)	5.5	20.0	18.8	35.7
Skeletal malformations				
Fusion of the frontal/parietal bones	0	1	0	2
Fissure of the parietal bone	0	0	3	0
Hypoplasia of the interparietal bone	0	1	0	0
Splitting of the parietal bones	0	0	3	1
Shortening of the nasal/frontal/mandibular bones	0	0	1	0
Hemivertebra	1	0	0	2
Unilateral ossification centre of the thoracic/lumbar vertebral bodies	0	1	0	0
Bifurcation of the ribs	1	0	0	0
Sternal cleft	0	0	1	0
Splitting of the sternebrae with sternocostal joint displacement	0	2	0	0
Total no. of foetuses with skeletal malformations	1	4	6	5
Percentage of foetuses with skeletal malformations (%)	0.7	3.1	4.0	4.5
Total no. of litters with skeletal malformations	1	3	2	5

Percentage of litters with skeletal malformations (%)	5.6	20.0	12.5	29.4
Variations				
No. of litters examined	18	15	16	14
No. of foetuses examined	140	130	150	112
No of litters with anomalous foetuses	16	14	16	8*
Percentage of litters with variations (%)	88.9	93.3	100	57.1
Skeletal variations				
No. of foetuses examined	140	130	150	112
27 presacral vertebrae	4	1	4	3
27 presacral vertebrae with 13th ribs	12	9	15	12
Cervical ribs	1	3	1	1
Lumbar ribs	23	19	41*	15
Extra ossification centre anterior to the 1st sternebra with costal cartilage joining	0	0	0	1
Total no. of foetuses with skeletal variations	40	32	61*	31
Total no. of litters with skeletal variations	16	12	16	8
Percentage of litters with skeletal variations (%)	88.9	80	100	57.1

* Significantly different from control at $p<0.05$.

Assessment and conclusion by applicant:
The study is considered acceptable, though treatment duration covered the period from implantation through organogenesis rather than through to the day prior to necropsy, as recommended in the current OECD 414 guideline. The NOAEL for maternal and developmental effects was 100 mg/kg bw/day and 300 mg/kg bw/day, respectively.

Assessment and conclusion by RMS:
The RMS agrees with the NOAEL set for maternal toxicity. The toxicological significance of the increased incidence of foetal malformations is considered unclear but in the absence of a clear dose-response and other the RMS agrees with a NOAEL at 300 mg/kg bw/day.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH).

B.6.6.2/12

Data point	CA 5.6.2/012
Report author	[redacted]
Report year	1993
Report title	Teratogenicity study in rabbits- Test compound: Glyphosate technical
Report No.	TOXI: 884-TER-RB
Document No.	Not available

Guidelines followed in study	OECD 414 (1981)
Deviations from current test guideline	Stated by the applicant: Dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy. Additional deviations noted by the RMS: • weight and histopathological changes • individual daya e.g. for clinical observations is not available of the thyroid glands of the dams, anogenital distance (AGD) in foetuses, • indication of incomplete testicular descent/cryptorchidism in male foetuses, • blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. • terms used for histopathological findings were not defined, e.g. dilated heart, sealshaped heart • due to the high mortalirty, less than16 animal with implantation sites were available precluding a poper assessment.
Previous evaluation	Yes, included in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Considered "supplementary" in the previous RAR. Taking into account the high mortality in high dose dams and thus the low number of foetuses available for assessment, the reliability of results is considered unclear. Therefore, the information is still considered "supplementary".

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: 60
Purity/Radiochemical purity: 96.8%
Vehicle: 0.5% aqueous carboxy methyl cellulose

Groups of presumed mated female New Zealand White rabbits were administered doses of 0, 20, 100 or 500 mg glyphosate/kg bw/day daily by gavage from day 6 to day 18 of pregnancy. Successful mating was considered day 0 of pregnancy. Dose levels were based on the findings of two preliminary studies.

In the first dose-range finding study, one male rabbit/dose was administered glyphosate technical by gavage at doses of 0 (control), 10, 20, 50, 500 or 1000 mg/kg bw/day for 13 days. Doses of ≥ 500 mg/kg bw/day resulted in reduced food intake, body weight loss and the dam administered 1000 mg/kg bw/day died on day 9 of treatment.

In a second dose-range finding study, one pregnant rabbit was administered 500 mg/kg bw/day glyphosate from day 6 to 18 and terminated on day 28 of gestation. Findings were compared with 20 historical control animals. There were no signs of toxicity and the body weight gain was greater (26 % more than the historical control mean) although feed intake was substantially reduced (34 % of historical control mean) and the litter size was reduced in the test female (4) compared with the historical control mean (7).

Main study
Animals were observed twice daily for onset and duration of pharmacotoxic symptoms and mortality. Body weights were recorded initially (before mating), on day 0, daily from day 6 through day 18 and on day 28, the day of sacrifice. Absolute body weight was derived by subtracting gravid uterine weight from day 28 body weight. The feed intake was recorded by weighing the food left on days 3, 6, 9, 12, 15, 19, 22, 25 and on day 28. Veterinary examination was made before and after mating and at the end of each week of experimental schedule.

A gross pathological examination was performed on all dams including animals found dead, moribund, and terminally sacrificed. On day 28 of pregnancy, dams were anesthetised, a cesarean section was made and the ovaries and uteri were excised and weighed and maternal and foetal data were recorded:

Maternal data:
Pregnant/non pregnant,uterine weight, number of corpora lutea, number of implantations, embryonic resorptions, fetal resorptions.

Fetal data:
Total number of fetuses, number of dead fetuses, number of abnormal fetuses, number of live fetuses and weight, umber of male fetuses and weight, number of female fetuses and weight, sex ratio. All the foetuses were examined for external, visceral and skeletal abnormalities. Structural changes were presented as variants, minor and major malformations.

RESULTS

MORTALITY
Two dams in the control group died due to mis-dosing whereas four mid dose dams and eight high dose dams died apparently as a consequence of treatment. The applicant considers the pathological examination to indicate that two of the deaths among high dose animals and one of the deaths in mid dose could be due to gavage errors (i.e. congestion in lung, trachea, froth in lung). However, the study author has categorised all deaths in mid and high dose animals treatment-related rather than accidental. Implantation sites were detected in 1/2, 2/4 and 6/8 control, mid and high dose dams that died during the study.

Table 6.6.2.012-1: Mortality data

Dose Group (mg/kg bw/day)	**0 (control)**	**20**	**100**	**500**
Mated females	26	17	16	15
Dead during treatment	1*	0	4	5
Died post-treatment	1*	0	0	3
Total number of deaths (historical control range 1-4)	2 (2)	0	4 (1)	8
% mortality	7.7	0.0	25.0	53.3

* Animal died due to mis-dosing
Parentheses indicate number of deaths stated to be due to gavage errors

CLINICAL SIGNS
Clinical signs of toxicity in the respiratory tract was observed in some high dose animals (dyspnoea, rales) and clinical signs from the gastro-intestinal tract (diarrhoea/soft stool and weakness) was observed in almost all of the high dose animals.

Table 6.6.2.012-2: Summary of relevant clinical signs in dams

Parameter / clinical sign	**0 (control)**	**20**	**100**	**500**
Mated females	26	17	16	16
Pregnant at termination	20	13	12	6
Rales	1	0	0	3
Soft stool with mucus	0	0	0	2
Soft stool/liquid faeces	0	0	1	12
Weak	0	0	0	2
Ocular discharge	0	0	0	1
Dyspnoea	0	0	0	1

BODY WEIGHT

The body weight was statistically significantly reduced in high dose animals and the terminal weight from which the uterine weight was subtracted was 10% lower than the concurrent controls. This reduction was not statistically significant and claimed to be similar to historical controls (data not included). The body weight gain in high dose animals was similar to controls. However, it should be noted that due to the high mortality, data in mid and high dose animals is based on a reduced number of animals.

Table 6.6.2.012-3: Summary of maternal body weight data (mean ± SD)

Parameter		Dose Group (mg/kg bw/day)			
	Historical positive control#	**0 (control)**	**20**	**100**	**500**
Number of dams pregnant at termination		20	13	12	6
Mean body weights (kg)					
Day 0		3.1 ± 0.5	2.8 ± 0.4	3.0 ± 0.2	2.6 ± 0.6* (↓16%)
Day 6		3.2 ± 0.5	3.0 ± 0.4	3.0 ±0.2	2.8 ± 0.7* (↓12%)
Day 18		3.2 ± 0.5	3.1 ± 0.5	3.1 ± 0.3	2.8 ± 0.7 (↓12%)
Day 28		3.3 ± 0.5	3.3 ± 0.4	3.3 ±0.3	3.0 ± 0.7* (↓9%)
Day 28 (body weight – uterine weight)	2.7*	3.0	3.0	2.9	2.7
Mean body weight gain (kg)					
Day 0 – 6 (Pre-treatment)	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
Day 6 – 18 (Treatment)	-0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.0 ± 0.2
Day 18 – 28 (Post treatment)	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.2
Day 0 – 28 (Throughout gestation)	0.3 ± 0.2	0.2 ± 0.3	0.5 ± 0.2**	0.3 ± 0.1	0.3 ± 0.3

Treatment with Acetylsalicylic acid (ASA) at 200 mg/kg bw/day
* Significantly lower than controls by Dunnett's test P≤0.05
** Significantly higher than controls by Dunnett's test P≤0.05

FOOD CONSUMPTION

There were no significant differences in food consumption between control and treatment groups except during treatment period (day 6-19) when food consumption was reduced by 31 % compared to concurrent controls.

Table 6.6.2.012-4: Summary of food consumption (mean ± SD)

		Dose Group (mg/kg bw/day)			
Parameter	**Historical positive control#**	**0 (control)**	**20**	**100**	**500**
Food consumption (g/rabbit/day)					
No of dams included in assessment	7	20	13	12	6
Day 0 – 6 (Pre-treatment)	105 ± 21.6	114 ± 31.8	88 ± 23.4*	125 ± 14.7	118 ± 25.4
Day 6 – 19 (Treatment)	70 ± 36.5*	103 ± 26.5	109 ±19.4	102 ±31.5	71 ± 56.9* (↓31%)

Day 19 – 28 (Post treatment)	129 ± 12.5	109 ± 38.7	135 ± 34.4	107 ± 40.1	105 ±25.4
Day 0 - 28	96 ± 22.5	107 ±27.3	113 ± 18.2	108 ± 24.4	92 ± 37.9

Treatment with Acetylsalicylic acid (ASA) at 200 mg/kg bw (treatment: day 6-18; post-treatment: day 18-28)
* Significantly lower than controls by Dunnett's test P≤0.05

REPRODUCTIVE PARAMETERS

There were no statistically significant differences in the number of corpora lutea, implantations, resorptions (embryonic and fetal) and pre or post-implantation losses between control and treated groups. However, complete resorptions were observed in one high dose dam but not in control or lower dose groups. The incidence of dams with any resorptions did not show any dose-response but again, a proper analysis is hampered by the high mortality.

Table 6.6.2.012-5: Summary of maternal observations

Dose group (mg/kg bw/day)					
	Historical positive control#	**0 (control)**	**20**	**100**	**500**
Mated females	12	26	17	16	15
Total number of deaths	4	2 (2)	0	4(1)	8 (5)
Pregnant at termination	7	20	13	12	6
Mean number of corpora lutea ± SD	9 ± 2.3	11 ± 2.8	10 ±2.4	10 ± 1.9	9 ± 2.0
Mean number of implantations ± SD	8 ±1.8	8 ± 2.0	8 ± 1.5	9 ± 1.8	6 ± 2.4
Total number of embryonic resorptions (%)	6 (11)	10 (7)	11 (11)	11 (11)	9 (24)
Total number of foetal resorptions (%)	2 (4)	8 (5)	7 (7)	13 (13)	1(3)
Total number of pre-implantation loss (%)	10 (19)	72 (48)	28 (29)	20 (20)	14 (37)
Total number of post-implantation loss (%)	8 (15)	18 (12)	18 (18)	24 (24)	10 (26)
Number of dams with any resorptions (%)	2 (29)	12 (60)	11 (85)	9 (75)	2 (33)
Dams with complete resorptions (%)	1 (14)	0 (0)	0 (0)	0 (0)	1 (17)

Treatment with acetylsalicylic acid (ASA) at 200 mg/kg bw

Because of the high mortality at 500 mg/kg/day (and thus, the reduced number of total litters), the total number of foetuses was substantially less in this dose group compared to the other dose groups. Therefore, parameters cannot be adequately assessed. Based on the data presented, the mean litter size, the mean numbers of abnormal, dead or live foetuses and the sex ratios of foetuses did not show any significant treatment-related differences.

Table 6.6.2.012-6: Mean litter data at caesarean section

	Historical positive control	**0 (mg/kg bw/day)**	**20**	**100**	**500**
Mated females	12	26	17	16	15
Total number of deaths	4	2	0	4	8
Pregnant at termination	7	20	13	12	6
Number of litters	6	20	13	12	5
Total number of foetuses	46	134	80	78	28
Mean litter size	8	7	6	7	6
Abnormal foetuses (%)	0 (0)	1 (1)	2 (3)	0	0
Dead foetuses (%)	0 (0)	0 (0)	1 (1)	1 (1)	0 (0)
Post-implantation loss (%)	8 (15)	18 (12)	18 (18)	24 (24)	10 (26)
Number of live foetuses	46	133	77	77	28
Mean weight of live foetuses (g ± SD)	29 ± 1.4	32 ± 5.3	35 ± 3.7* (↑21%)	35 ± 2.4* (↑21%)	33 ± 4.9
Sex ratio (Male : Female)	1 : 1.3	1 : 0.7	1 : 1.2	1 : 1.2	1 : 1.8

# Treatment with acetylsalicylic acid (ASA) at 200 mg/kg bw
SD = standard deviation

The foetal body weights were statistically significantly increased in the 20 and 100 mg/kg/day dose groups but in the absence of a clear dose-response, this is not considered an adverse effect.

There was no increased incidence of major external malformations but a major visceral malformation primarily affecting the heart was observed although in a single incidence and without dose-response. However, dilated heart, was reported in four foetuses of 3 litters in the 20 mg/kg bw/day dose group, 4 foetuses (3 + 1) from 2 litters of the 100 mg/kg bw/day dose group and all foetuses (4) of one litter and one foetus of another litter at the 500 mg/kg bw/day. According to the applicant, *"the terminology used to describe the heart malformations in this study is different from that typically employed in teratology research (e.g., dilated heart, seal-shaped heart). Consequently, what is meant by the description "dilated heart" is not well defined and not documented with photographs or retained tissue sections or slides. How this malformation might relate to others reported in the heart (i.e., dilated left or right ventricle, seal-shaped heart, cardiomegaly) is not clear. Further, because too few foetuses were available for examination in the high dose group, it cannot be determined whether these defects exhibited a true dose-related increase. It is important to note, however, that only 2 litters exhibited major visceral malformations in the high dose group. Additionally, these findings were found in the presence of extensive maternal toxicity, evidenced by reduced food consumption and body weight gains in the few animals that survived this dose level, clinical signs, and substantial deaths."*

This finding was discussed by RAC in the context of classification and labelling: "*RAC concludes that the high incidence of maternal deaths is considered to lead to an insufficient number of foetuses being available for assessment from the high dose group (i.e 28 fetuses from 5 litters). Further, RAC considers that the reporting of cardiovascular malformations was insufficient due to a lack of measurements of the heart and that no definition of the diagnosis was provided in the study report. No information regarding the historical control data for dilated heart was included by the DS or provided in the study report."* (CLH-O-0000001412-86-149/F, Adopted 15 March 2017).

Table 6.6.2.012-7: Cardiovascular malformations in the rabbit study (reproduced from RAC opinion)

Dose group (mg/kg bw/d)	0	20	100	500
No. of foetuses/no. of litters exmined	133/20	78/13	77/12	28/5
Major visceral malformations:				
No. of foetuses/litters with dilated heart	-	4*/3	4*/2	5*/2*
No. of foetuses/litters with cardiomegaly	0	0	1**	0
No. of foetuses/litters with “seal shaped” hearts	1/1	0	1**	0
No. of fooetuses/litters with dilated ventricle	1/1	0	1/1	1/1
No. affected/total no. of foetuses	2/133	4/78	4/77	5/28
Litters affected/total no. of litters	2/133	3/13	2/12	2/5

* statistically significant, $p \leq 0.05$
** same foetus

The percentage of foetuses with extra 13th rib increased with increasing dose. The incidence in historical controls was much higher but this data seems less reliable considering the incidences in concurrent controls and low dose animals.

Table 6.6.2.012-8: Summary of relevant external, visceral and skeletal findings (litter data)

Foetal findings		Dose level (mg/kg bw/day)			
Data#	**HC**	**0**	**20**	**100**	**500**
No. of litters examined	6	20	13	12	5
No. of foetuses examined	46	133	79	77	28
Minor external malformations					
Percentage of small foetuses (%)	--	0	0	1.3	0
Litter incidence (%)	--	0	0	8.3	0
Major external malformations					
Percentage of foetuses with upper cleft palate (%)	0	0.8	2.5	0	0
Litter incidence (%)	0	5	15	0	0
Percentage of foetuses with forelimb arthrogryposis	--	0	0	1.3	0
Litter incidence (%)	--	0	0	8.3	0
Percentage of	0	0.8	2.5	0	0

foetuses with multiple malformations					
Litter incidence (%)	0	5	15	0	0
Percentage of foetuses with major malformations (%)	0	1.5	2.5	1.3	0
Litter incidence (%)	--	10	7.7	8.3	0
Major visceral malformations					
Percentage of foetuses with dilated heart (%)	--	0	5.1*	5.2*	17.9*
Litter incidence (%)	--	0	23.1	16.7	40.0
Percentage of foetuses with anencephaly (%)	0	0.8	0	0	0
Litter incidence (%)	0	5.0	0	0	0
Percentage of foetuses with heart–seal shaped (%)	0	0.8	0	0	0
Litter incidence (%)	0	5.0	0	0	0
Percentage of foetuses with cardiomegaly & sealed heart (%)	--	0	0	1.3	0
Litter incidence (%)	--	0	0	8.3	0
Percentage of foetuses with dilated ventricle (left) (%)	--	0	0	1.3	0
Litter incidence (%)	--	0	0	8.3	0
Percentage of foetuses with dilated ventricle (right) (%)	--	0	0	0	3.6
Litter incidence (%)	--	0	0	0	20
Percentage of foetuses with persistent truncus arteriousus (%)	--	0.8	0	0	0
Litter incidence (%)	--	5.0	0	0	0
Percentage of	--	0	0	0	3.6

foetuses with gallbladder absent (%)					
Litter incidence (%)	--	0	0	0	20
Percentage of foetuses with liver (median) haematoma (%)	--	0	0	0	3.6
Litter incidence (%)	--	0	0	0	20
Minor skeletal malformations No. of foetuses with extra 13th rib		0	1	2	1
Percentage of foetuses with extra 13th rib	8.7**	0	1.3	2.6	3.6*
Litter incidence (%)	--	0	7.7	16.7	20
Major skeletal malformations Percentage of foetuses major malformations (%)	10.9	8.3	6.3	0*	3.6
Litter incidence (%)	50	20	23.1	0	20

Historical positive control data (--: no data available)
* Significantly different from control at $p < 0.05$.
** Significantly different from control by Contingency test ($P \leq 0.05$)

Assessment and conclusion by applicant:
The study is considered invalid due to numerous weaknesses including a small number of litters for examination (low pregnancy rate at all dose levels, lethality in mid and high dose dams), high apparent gavage error rate resulting in mortality, and other reporting deficiencies. The maternal mortality rate for deaths not attributable to garage errors falls within the range of historical control mortalities from this laboratory. The percentage of foetuses with 'dilated heart' was significantly increased at all dose levels. However, the absolute number of affected foetuses and litters is quite small and did not show a marked difference between the treated groups. Unfortunately, the reporting of "dilated heart" in the study report is not consistent Appendix 35 "Protocol for Visceral Evaluation" in this report, which notes four different categories of dilated heart; (i) dilation of the ventricle of the olfactory bulb, (ii) dilation of the lateral ventricle, (iii) dilation of the third ventricle, and (iv) dilation of the fourth ventricle. Given the reporting is not consistent with the detail of the protocol, a low level of confidence is placed in the reporting of these different endpoints.The diagnosis 'dilated heart' was not defined in this study report, does not follow "Appendix 35 Protocol for Visceral Evaluation", and neither criteria used for this diagnosis nor measurements of the heart were provided. In addition, this group of reported malformations lacks a clear dose-response noting that litters, not foetuses, are the experimental unit in developmental toxicity studies; dilated heart foetuses (litters) were 0 (0), 4 (3), 4 (2), and 5 (2) across 0, 20, 100 and 500 mg/kg bw/day, respectively. The inconsistency of malformation terminology in this study report when compared both with other studies and this laboratory's own visceral evaluation terminology in Appendix 35, suggests that interpreting this group of malformation types within this study is not appropriate or justified. Given the inaccuracies in reporting of heart findings and lack of dose-response, the offspring NOAEL in disregarding these endpoints, should be 100 mg/kg bw/day, due to the high dose proving to be well in excess of a limit dose. Similarly, the maternal NOAEL is 100 mg/kg bw/day, since the maternal mortality at this dose is within the range of historical controls for this laboratory. However, little if any weight should be placed on this study due to its many shortcomings. The applicant believes this study should be disregarded as unreliable for the above mentioned reasons.

Assessment and conclusion by RMS:
The NOAEL for maternal toxicity is considered 20 mg/kg bw/day based on excessive mortality at higher dose levels and clinical signs of toxicity (reduced food consumption, soft faeces, reduced body weight gain during the dosing period and one incidence of complete resorptions at the 500 mg/kg bw/day dose level). A NOAEL for developmental toxicity cannot be set since the low number of foetuses available precludes a robust assessment.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH).

B.6.6.2/13

Data point	CA 5.6.2/013
Report author	[redacted]
Report year	1993
Report title	Amendment to final report - Teratogenicity study in rabbits – Test compound: Glyphosate technical (FSG 03090 H/05 March 1990)
Report No.	TOXI: 884-TER-RB
Document No.	Not available
Guidelines followed in study	See above
Deviations from current test guideline	See above
Previous evaluation	See above
GLP/Officially recognised testing facilities	See above
Acceptability/Reliability	See above

The document contains a statement concluding "*Considering the results the No Observed Adverse Effect Level (NOAEL) of Glyphosate Technical for embryo/fetotoxic and teratogenic effect in New Zealand White rabbits appears to be less than 20 mg/kg body weight (the minimum test dose used) under the test conditions and dose employed".*

Please note the RMS conclusions for study 5.6.2/012.

B.6.6.2/14

Data point	CA 5.6.2/014
Report author	[redacted] *et al.*
Report year	1991
Report title	The Effect of Glyphosate on Pregnancy of the Rabbit (Incorporates Preliminary Investigations)
Report No.	[redacted] 45 & 39 (preliminary study with pregnant does) & 40 (preliminary study with non-pregnant does) /901303
Document No.	Not reported
Guidelines followed in study	OECD 414 (1981), US EPA 83-3
Deviations from current test guideline	Stated by the applicant: Dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy. Additional deviations noted by the RMS: • weight and histopathological changes of the thyroid glands of the dams, uterine weight, anogenital distance (AGD) in foetuses, • Fetal body weight by sex was not reported

	• indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.
Previous evaluation	Yes, accepted in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Acceptable

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: 206-JAK-25-1
Purity/Radiochemical purity: 98.6%
Vehicle: 1 % methylcellulose

Based on results from two preliminary studies (in pregnant and non-pregnant rabbits, respectively) showing mortality, gastrointestinal disturbances, reduced body weight gain and food consumption, doses of 0, 50, 150 or 450 mg glyphosate/kg bw was administered groups of 16 - 20 time-mated female New Zealand White rabbits daily from day 7 to day 19 of pregnancy using gavage. The day of mating was considered as day 0. The doses were chosen based on the results of a pilot study and a preliminary study.

All animals were regularly handled and observed daily for overt changes or signs of reaction to treatment. Animals that died or were killed for animal welfare reasons were weighed and subjected to post-mortem examination. Individual body weights were recorded on days 1, 7, 9, 11, 15, 20, 24 and 29 of gestation and food consumption was recorded on days of weighing throughout gestation.

All surviving does were sacrificed on day 29 of pregnancy and subjected to post-mortem examinations for congenital abnormalities and gross pathological changes in maternal organs. The ovaries and uteri were examined to determine the number of corpora lutea, the number and distribution of live young, the number and distribution of embryonic and /foetal deaths, individual foetal weight and foetal abnormalities. Embryonic/foetal deaths were classified as early, late or abortions. Live young were examined for external, visceral and skeletal abnormalities and after termination examined for visceral abnormalities. Where appropriate, suspected abnormalities were further examined by alternative procedures such as microdissection and histopathology to clarify initial observations. Pups were fixed, the heads sliced along the line of the frontoparietal suture and the brain examined for abnormalities before clearing and staining the carcasses for skeletal examination. Structural changes were presented as malformations, anomalies or variants.

RESULTS

MORTALITY
One high-dose dam died on day 20 following signs of abortion on day 19 and signs of gastrointestinal disturbance, (i.e. soft/liquid faeces, severe reduction in food consumption and body weight loss observed from the onset of treatment. Necropsy findings included pale heart and kidneys, a few haemorrhagic depressions in the stomach and findings confirming the abortion (3 late embryonic deaths and 7 abortion sites) and the disturbances in the gastrointestinal tract (fluid contents in small intestine, watery distention in caecum, minimal soft contents in colon). Two other deaths (a broken hind leg in a high dose animal before initiation of study and an incidence of congenital abnormality in a control dam on day 29) were eliminated from the study assessment.

CLINICAL OBSERVATIONS
Clinical signs included a dose-related increase in the number of females showing soft/liquid faeces and signs indicating a lack of appetite (off feed/reduction in food consumption) at 150 and 450 mg/kg bw/day (see below).

Table 6.6.2.014-1: Summary of maternal performance and relevant clinical signs

Parameter				
Dose Group (mg/kg bw/day)	**0 (control)**	**50**	**150**	**450**
Mated females	19	19	16	20
Not pregnant	0	6	1	5
Number of does with live young or litters at day 29	18	12	15	13
Clinical signs (only animals with live young included)				
Off-feed	8	6	10	9
Reduced faecal output	9	8	11	12
Soft/liquid faeces	0	2	5	13

FOOD CONSUMPTION

The food consumption was slightly reduced by 12% during days 11 – 19 in animals receiving 150 mg/kg bw/day and by 6-17% throughout the treatment period (days 7-19) in the 450 mg/kg bw/day dose group. Changes were not statistically significant.

Table 6.6.2.014-2: Summary of mean food consumption (g/rabbit/day)

Dose Group (mg/kg bw/day)				
	0 (control)	**50**	**150**	**450**
Mated females	19	19	16	20
No. of animals included in assessment	18	12	15	13
Food consumption (g/rabbit/day) during				
Days 1-6	142	143	141	152
Days 7-8	143	154	150	135
Days 9-10	146	148	148	132
Days 11-14	153	149	134	129
Days 15-19	148	151	131	123
Days 20-23	142	154	149	149
Days 24-28	131	143	153	166

BODY WEIGHT

The reduced food consumption seemed associated with a slightly reduced body weight gain observed from day 11 of pregnancy to termination of treatment in the 150 and 450 mg/kg bw/day dose groups.

Table 6.6.2.014-3: Summary of body weight data (group means)

Parameter			**Dose Group (mg/kg bw/day)**	
	0 (control)	**50**	**150**	**450**
Mated females	19	19	16	20
No of animals included in assessment	18	12	15	13
Body weights (g) at				
Day 1	3538	3524	3568	3658
Day 7	3582	3604	3624	3709
Day 9	3589	3639	3637	3732
Day 11	3601	3653	3661	3743
Day 15	3742	3804	3779	3833
Day 20	3770	3831	3775	3835
Day 24	3844	3927	3849	3965
Day 29	3999	4084	3975	4103

Bwg (day 1-29)	461	560	407	445 (↓3%)
Bwg (day 11-29)	398	431	314 (↓21%)	360 (↓10%)

PATHOLOGY
The post mortem examination did not indicate any findings that seemed related to treatment.
Since the number of pregnant dams was lower in the high dose group, less foetuses were available for the assessment. Moreover, total litter loss occurred in the high dose female which aborted on day 19 and died. However, the litter size at caesarean necropsy was comparable in all treatment groups.

There were no statistically significant intergroup differences in the numbers of corpora lutea, implantations, pre-implantation loss, foetal sex ratios or foetal weights. There was a statistically significant increase in embryo/foetal death and post-implantation loss in all treated groups. The study author did not consider these observations biologically significant in the absence of a clear dose-response, since the control value was at the lower end of the historical control range (from 21 studies performed at these laboratories Jan. 89-June 90) and the exposed groups were at the expected or slightly above the expected level. Although embryo/foetal death was within the historical control range, post-implantation loss was above the historical control values in the high-dose group, and both of these parameters were statistically significant ($p<0.01$) at the high dose. Nevertheless, the data for the different groups in this experiment as well as in the historical control data indicate a large variation in post-implantation loss and it is thus difficult to interpret.

Table 6.6.2.014-4: Summary of the maternal and litter parameters (group mean values)

Dose Group (mg/kg bw/day)	**0 (control)**	**50**	**150**	**450**	**Historical control range (mean value)**
No. of mated females	19	19	16	20	--
No. not pregnant	0	6	1	5	--
No. of does with live young or litters at Day 29	18	12	15	13	--
Corpora lutea	11.5	12.4	11.7	11.3	9.0 – 12.9 (11.2)
Implantations	9.7	10.5	9.0	9.2	7.0 – 11.1 (9.5)
Pre-implantation loss	14.6	15.4	23.4	18.8	2.3 – 26.1 (15.1)
Early embryonic deaths	0.4	0.9	0.9	0.5	0.3 – 1.1 (0.6)
Late embryonic deaths	0.2	0.9	0.5	1.3**	0.1 – 1.3 (0.7)
Abortions	0.0	0.0	0.1	0.0#	0.0 – 0.1 (0)
Total embryonic deaths	0.6	1.8*	1.5*	1.8**	0.6 – 2.0 (1.2)
Post-implantation loss (%)	5.7	19.5*	15.3*	21.0**	6.5 – 17.5 (12.9)
Live young	9.1	8.7	7.5	7.3	6.1 – 9.5 (8.3)
Litter weight (g)	389.5	370.6	320.5	315.0	281.9 – 402.2 (352.9)
Mean foetal weight (g)	43.9	43.3	44.0	44.5	41.4 – 47.6 (44.1)
Sex (% males)	55.3	55.8	57.6	53.8	--

* Statistically significant by Kruskal –Wallis 'H' test $P<0.05$
** Statistically significant by Kruskal –Wallis 'H' test $P<0.01$
# Fisher exact test follow-up by intergroup comparison with control was not statistically significant $P>0.05$

The incidence of malformations was slightly increased in mid and high dose groups compared to controls and appeared located in the pulmonary/cardiac tract. Although the total number of malformations (foetuses and affected litters) was not outside the historical control range reported and values were not statistically different from concurrent control values, the incidence of each type of malformations noted (i.e. interventricular septal defect, enlarged left, reduced right ventricles, retro-oesophageal right subclavian artery and narrow/dilated aortic arch/pulmonary trunk/arterial trunk) was outside the historical control range for the effect. According to the applicant, *"several of the cardiovascular malformations that were observed, particularly in the high-dose group, occurred in the same animals and are related to a single morphogenetic mechanism (i.e., displacement of the developing aorticopulmonary septum), which is likely to adjust during the first two weeks of postnatal life. These related findings, which often cluster together, included dilated/narrow aorta and narrow/dilated pulmonary artery; interventricular septal defect; and disproportionately sized right and left ventricles. These findings were observed (often in clusters) in the historical control data that were provided by the conducting laboratory. Individual presentation of these malformations in tables when the malformations occurred together in the same foetus and are due to the same mechanisms and artificially inflates the sense that there is a much stronger cardiac effect than is actually present."*

The RMS questions this conclusion since although several malformations of the heart were observed in one high dose foetus, there were several other malformations of the heart observed in five additional high-dose foetuses and in 6 different mid-dose foetuses.

The applicant further states that *"The cardiac malformation observed with greatest frequency in this study was interventricular septal defect. The number of foetuses and litters with ventricular septal defects were 1, 1, 1 and 4 in the 0, 50, 150 and 450 mg/kg bw/day dose groups, respectively. Comparison of the historical control data (see table below) shows that the heart findings (when presented on a percent individual and/or litter incidence basis) were slightly outside of the historical background range from 13 studies conducted during the same period. However, the disparity in values is a consequence of the small numbers of litters in the study report. If the data are displayed as a fraction (rather than a percentage), then the number of litters affected were 1/18, 1/12, 1/15, and 4/13 in the 0, 50, 150, and 450 mg/kg/day dose groups, respectively. The historical control range is 0/19 – 3/13. Thus, the findings at the high dose are barely outside of the historical control range. Further, they were observed in conjunction with clear signs of maternal toxicity (reduced food consumption, body weight gains and increased clinical signs).The other cardiovascular finding found in this study not related to the morphogenetic mechanism involving formation of the spiral septum is retroesophageal right subclavian artery. This finding was also observed regularly throughout the historical period. It is not uncommon and is oftentimes an inconsequential anatomical difference in vascular arrangement. At autopsy this condition is found in 0.5 – 2.0 % of subjects."*

The RMS questions also this conclusion since maternal toxicity was not excessive (mortality of one dam following gastrointestinal disturbances and a 10% non-statistically significant reduction of body weight gain) thus such association seems less clear. The study and the effect were assessed in terms of classification and labelling in 2017:
The RAC opinion states: *"Interventricular septal defects were recorded at the highest dose, and were seen in 4 foetuses from 4 litters (i.e. at an incidence outside the historical control data). The same effects were seen in one foetus from each of the other dose groups, including the control group. Other cardiovascular malformations of low incidence (but still outside the historical control data) were; enlarged left ventricles, reduced right ventricles, retro-oesophageal right subclavian artery and narrow/dilated aortic arch/pulmonary trunk/arterial trunk. It should however, be noted that in the high dose group interventricular septal defect, enlarged left, reduced right ventricles and narrow/dilated aortic arch/pulmonary trunk/arterial trunk originated from two foetuses from two different litters. Retro-oesophageal right subclavian artery was reported in two foetuses from the same litter, one of these foetuses were also reported to have interventricular septal defect. Thus, the cardiovascular malformations were to some extent clustered together in the same foetuses. In the mid-dose group all three foetuses with retro-oesophageal right subclavian artery were from the same litter (see table below). Maternal toxicity was reported as one maternal death at the top dose of 450 mg/kg bw/d on GD 20 following abortion, GI disturbances, reduced food intake and body weight loss. Females in the two highest dose groups showed reduced food consumption compared to the controls, but these were not statistically significant. In the mid-dose at 150 mg/kg bw/d a reduction of 12% was observed from GD 11-19. At 450 mg/kg bw/d this was also evident throughout the treatment period with reductions of 6-17 % during GD 7-19. No changes in maternal bw throughout gestation were reported. A dose related increase in females showing soft/liquid faeces and signs of lack of appetite were seen at the two highest doses. However, in the top dose group there was no*

clear correlation between the severity of the maternal toxicity and the fetuses with interventricular septal defects. RAC concludes that the reported increase in cardiovascular malformations were to some extent clustered together in the same fetuses and was shown in the presence of maternal toxicity, however, it was not considered marked."

There were no treatment-related skeletal malformations or variations.

Table 6.6.2.014-5: Summary of foetal examination

Dose Group (mg/kg bw/day)	**0 (control)**	**50**	**150**	**450**	**Historical control range or x/y □ (mean)**
Number of does with live young or litters at Day 29	18	12	15	13	--
Mean foetal weight (g)	43.9	43.3	44.0	44.5	41.4 – 47.6 (44.1)
Sex (% males)	55.3	55.8	57.6	53.8	--
Malformations			--		
Total number of foetuses examined	163	104	112	95	1511
No. of malformed foetuses	3	3	5	6	51
%	1.9	5.8	4.3	5.9 (F)	0.7 – 5.9 (3.8)
Number of Affected Litters	3	3	3	5	43/188
%	16.67	25	20	38.5	22.9
Thoracic region malformations			--		
No. of foetuses with interventricular septal defect	1	1	1	4	10/1511
%	0.6	1.0	0.9	4.2	0.66
Litter incidence	1	1	1	4	10/188
%	5.56	8.3	6.67	30.8	5.32
Foetuses with enlarged left, reduced right ventricles	0	0	0	2	2/1511
%	0.0	0.0	0.0	2.1	0.13
Litter incidence	0	0	0	2	2/188
%	0	0	0	15.4	1.10
Foetuses with retro-oesophageal right subclavian artery	0	0	3	2	7/1511
%	0.0	0.0	2.7	2.1	0.46
Litter incidence	0	0	1	1	7/188
%	0	0	6.6	7.6	3.72
Foetuses with narrow/dilated aortic arch/pulmonary trunk/arterial	1	1	1	3	8/1511

trunk					
%	0.6	1.0	0.9	3.2	0.52
Litter incidence	1	1	1	3	8/188
%	5.56	8.3	6.67	23.1	4.25
Anomalies			--		
Total number of foetuses examined#	160	101	107	89	--
No. of foetuses with gross/visceral anomalies	9	14	14	6	--
%	6.4	19.5	12.9	9.6 (K)	--
No. of foetuses with skeletal anomalies	21	13	14	11	--
%	11.7	17.7	12.5	10.1 (K)	--
No. of foetuses with reduced ossification	7	4	5	4	--
%	4.4	4.0	4.7	4.5	--
Mean foetal weight of foetuses with reduced ossification (g)	37.9	43.6	37.7	26.1	--

#..Total number at caesarean section
Number ± SD; historical control without SD
 number affected / total number examined
Malformed foetuses are excluded
(F) Fisher's exact test applied, not statistically significant (P>0.05)
(K) Kruskal-Wallis 'H' statistic, not significant (P>0.05)
-- no data

Assessment and conclusion by applicant:
The study is considered acceptable, though treatment duration covered the period from implantation through organogenesis rather than through to the day prior to necropsy, as recommended in the current OECD 414 guideline. In this study, there was a significant increase in embryonic death and post-implantation loss in treated groups compared to controls, however without a clear dose-relationship. Regarding the post-implantation losss, values for the low and high dose groups are outside the historical control range. The cardiac malformation observed with greatest frequency in this study was the interventricular septal defect. At 450 mg/kg bw/day this effect was outside the historical control range (4.2 % compared to 0.66 % in historical controls). Taken into account the high post implantation loss at the same dose level, the incidence of additionally cardiac malformation may be covered. At mid dose level, foetuses with an higher incidence of retrooesophageal right subclavian artery were reported. However, this effect has to be considered equivocal, because no clear-dose relationship could be established.

In conclusion, the NOAEL for maternal toxicity is considered 50 mg/kg bw/day based on slightly reduced food consumption, slightly reduced body weight gain and soft/liquid faeces at 150 mg/kg bw/day. The NOAEL for developmental toxicity is considered to be 150 mg/kg bw/day based on the post implantation loss, late embryonic death and an increase in cardiac malformations at 450 mg/kg bw/day.

Assessment and conclusion by RMS:
The RMS agrees with the NOAELs proposed by the applicant.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete

testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH). Uterine weight.

B.6.6.2/15

Data point	CA 5.6.2/015
Report author	[redacted] *et al.*
Report year	1991
Report title	Historical control data: Control Indiviual Incidence Major Anormalities (Interfauna Rabbit 1989)
Report No.	[redacted] 45 & 39 & 40/901303
Document No.	Not reported
Guidelines followed in study	See above
Deviations from current test guideline	See above
Previous evaluation	See above
GLP/Officially recognised testing facilities	See above
Acceptability/Reliability	See above

B.6.6.2/16

Data point	CA 5.6.2/016
Report author	[redacted]
Report year	1989
Report title	Rabbit Teratology Study with Glyphosate Technical
Report No.	1086
Document No.	Not reported
Guidelines followed in study	OECD 414 (1981)
Deviations from current test guideline	Stated by the applicant: Group size smaller than required, dosed from day of implantation through the period of organogenesis, rather than through to day prior to necropsy, no uterine weight reported, no maternal necropsy findings reported. According to the applicant, deviations from the current version of OECD 414 (2018) are basically due to the fact that the study was aligned to an older version of the OECD test guideline 414. Additional deviations noted by the RMS: • Statistical analyses were not made.. • Individual data is not included. • Weight and histopathological changes of the thyroid glands of the dams, uterine weight not presented, anogenital distance (AGD) in foetuses, • Indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected.
Previous evaluation	Considered “supplementary” in RAR (2015)
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Non-acceptable (considered “supportive” by applicant) See RMS assessment and conclusions.

MATERIALS AND METHODS

Test material:	Glyphosate Technical
Lot/Batch:	38
Purity/Radiochemical purity:	95%
Vehicle:	0.1% gum acacia in water

Doses of 0, 125, 250 and 500 mg/kg bw/day glyphosate in 0.1% gum acacia in water were administered groups of 15 New Zealand White female rabbits by gavage from gestation day 6-18 (the day of mating was recorded as the 1st day of pregnancy).

Clinical signs
Animals were checked twice daily (before and after dosing) during the dosing period for signs of toxicity, ill-health or behavioural changes and individual body weights and food consumption were recorded on days 0, 6, 12, 18, 23, and 29 (at necropsy). Females were sacrificed on day 29 of gestation and examined for any abnormalities that would affect pregnancy. The ovaries and uteri were removed, the uterine weight was recorded and the ovaries were examined for the number of corpora lutea and uteri for the number and position of implants and dead or live foetuses. Uteri from non-gravid females were placed in 10% ammonium sulfide solution for detection of early resorptions. Each rabbit foetus was removed, all live foetuses were weighed and examined for external malformations including cleft palate and variations. All live foetuses were examined for thoracic and visceral abnormalities, and each foetus was sexed. Following visceral examination, all foetuses were eviscerated and processed for skeletal staining and heads were fixed and examined for changes in craniofacial structures.

RESULTS

The mean food consumption was similar to controls in low and mid dose animals but reduced by 16-18% in high dose group starting with the day of treatment throughout the rest of the observation period. This was accompanied by a reduced body weight gain of 28-35% during treatment and by 41% during post-exposure.

Table 6.6.2.016-1: Food consumption (mean ± SD)

Dose Group (mg/kg bw/day)	**0 (control)**	**125**	**250**	**500**
Food consumption (g/rabbit/day)				
No of dams included in assessment	15	15	15	15
Day 0 – 6 (Pre-treatment)	92.3 ± 8.55	97.2 ± 6.56	96.7 ± 6.73	100.1 ± 6.32
Day 6 – 12 (Treatment)	98.7 ± 6.53	99.5 ± 5.76	100.7 ± 6.30	82.7 ± 6.61 (↓16%)
Day 12 – 18 (Treatment)	98.6 ± 6.62	98.4 ± 4.34	94.4 ± 4.58	80.9 ± 4.87 (↓18%)
Day 18 – 29 (Post treatment))	192.5 ± 7.69	202.5 ± 8.93	205.3 ± 7.07	162.1 ± 12.03 (↓16%)

Table 6.6.2.016-2: Maternal body weight changes (mean ± SD)

Dose Group (mg/kg bw/day)	**0 (control)**	**125**	**250**	**500**
Body weight change (g)				
No of dams included in assessment	15	15	15	15
Day 0 – 6 (Pre-treatment)	166.5 ± 3.35	160.5 ± 12.15	153.6 ± 19.91	156.3 ± 9.15
Day 6 – 12 (Treatment)	14.0 ± 5.41	8.0 ± 5.09	13.0 ± 7.06	10.1 ± 7.08 (↓28%)
Day 12 – 18	61.7 ± 21.07	73.0 ± 18.11	69.5 ± 18.45	40.0 ± 16.15

(Treatment)				(↓35%)
Day 18-29 (Post exposure)	116.7 ± 39.56	125.9 ± 25.51	109 ± 22.44 (↓7%)	68.9 ± 15.99 (↓41%)

Two high dose dams aborted and consequently had no live foetuses. The mean number of corpora lutea, mean number of total implants per litter, mean percentage of pre-implantation loss, and mean number of early resorptions were similar between control and treated groups. The mean number of viable and non-viable implants (foetuses) per litter was lower in the high dose group but no statistical comparisons were provided in the report. The sex ratio seemed slightly shifted towards a higher percentage of male foetuses and the mean foetal bodyweights were increased in treated animals. However, as stated above, no statistical analyses were made.

Table 6.6.2.016-3: Gestational parameters and foetal weights

Dose level (mg/kg bw/day)	**0**	**125**	**250**	**500**
No. of mated females	15	15	15	15
No. of early deliveries	0	0	0	0
No. of abortions	0	0	0	2
No. of females without foetuses	0	0	0	2
No. nonpregnant at termination	2	1	1	3
No. of litters	13	14	14	12
Mean no. of corpora lutea per doe ± SD	10.0 ± 1.69	10.1 ± 1.60	10.3 ± 1.44	9.8 ± 1.57
Mean no. of total implants per litter ± SD	9.0 ± 1.20	9.3 ± 1.33	9.4 ± 1.12	8.5 ± 1.05
Mean % pre-implantation loss ± SD	21.3 ± 32.4	14.9 ± 24.09	14.7 ± 24.38	13.1 ± 6.34
Mean no. of viable implants per litter ± SD	7.3 ± 3.10	8.0 ± 2.59	8.0 ± 2.59	5.2 ± 3.03
Mean no. of non-viable implants per litter ± SD	0.07 ± 0.26	0.13 ± 0.35	0.27 ± 059	1.4 ± 2.20
Mean no. of early resorptions per litter ± SD	1.7 ± 3.22	1.1 ± 2.53	1.0 ± 2.56	1.9 ± 2.43
Sex ratio (% males) ± SD	44.4 ± 20.17	49.2 ± 6.90 (↑ 11%)	49.7 ± 11.45 (↑ 12%)	50.1 ± 13.00 (↑ 13%)
Mean foetal body weight per litter ± SD	40.6 ± 16.6	47.1 ± 0.95 (↑ 16%)	47.5 ± 1.38 (↑ 17%)	48.7 ± 1.87 (↑ 20%)

The incidences of visceral and skeletal malformations were higher in treated animals compared to controls. With respect to heart malformations, incidences of 0, 1, 1, and 2 interventricular septal defects were observed in the 0, 125, 250, and 500 mg/kg bw/day dose groups. Increased incidences of smaller than normal size of right ventricle and globular heart was observed in treated animals compared to controls. The incidence of abnormal tail, absent kidney and absent postcaval lung lobe was increased in high dose animals.

Table 6.6.2.016-4: Incidence of foetal malformations and variations

Dose level (mg/kg bw/day)	**0**	**125**	**250**	**500**
Malformations				
No. of litters examined	13	14	14	12
No. of foetuses examined	109	113	120	78
No of litters with malformations	3	6	10	12
% of litters with malformations	23.08	42.86	71.43	100
No. of foetuses with malformations	3	6	10	20
% of foetuses with malformations	2.75	5.31	8.33	25.64
Number of foetuses (litters) with **external malformations**				
Tail abnormal	1 (1)	1 (1)	2 (2)	3 (2)
Low-set ears	0 (0)	1 (1)	1 (1)	2 (1)
Total external malformations	1	2	3	3
Total external malformations (%)	0.92	1.77	2.50	3.85
Number of foetuses (litters) with **visceral malformations**				
Ventricular septal defect	0 (0)	1 (1)	1 (1)	2 (2)
Postcaval lung lobe absent	0 (0)	1 (1)	2 (2)	4 (3)
Kidney(s) absent	1 (1)	2 (2)	2 (2)	6 (4)
Total visceral malformations	1	4	5	12
Total visceral malformations (%)	0.92	3.54	4.17	15.38
Number of foetuses (litters) with **skeletal malformations**				
Rudimentary rib (no. 14)	1 (1)	0 (0)	2 (2)	5 (2)
Total skeletal malformations	1	0	2	5
Total skeletal malformations (%)	0.92	0.00	1.67	6.41
Variations				
No. of foetuses examined	109	113	120	78
Total no. of observed variations	26	30	49	93
Number of foetuses (litters) with **external variations**				
Tail blunt tipped	1 (1)	0 (0)	3 (2)	5 (4)
Number of foetuses (litters) with **visceral variations**				
Irregular rugae on palate	0 (0)	2 (1)	3 (2)	2 (2)
Lateral ventricles of cerebrum dilated	0 (0)	2 (2)	2 (2)	6 (4)
Right ventricle small than normal	1 (1)	3 (2)	3 (2)	5 (3)
Globular heart	2 (2)	0 (0)	3 (2)	5 (4)
Incomplete	1 (1)	2 (1)	2 (1)	4 (2)

separation of lung lobes				
Parietal foetal atelectasis	0 (0)	1 (1)	1 (1)	1 (1)
Liver irregular shape	0 (0)	2 (1)	2 (2)	6 (4)
Kidney(s) globular shape	0 (0)	0 (0)	2 (1)	5 (3)
Number of foetuses (litters) with **skeletal variations**				
Cervical centra 1-3 and/or 4 bilobed	1 (1)	0 (0)	1 (1)	2 (2)

Also the number of variations observed was increased in the high dose group. The applicant argues that the increase in malformations and variations observed in the high dose group occurred in the presence of maternal toxicity (reduced food consumption and body weight gains). Further, this was at a dose (500 mg/kg bw/day) that caused significant toxicity, including mortality, in another rabbit developmental study. The RMS agrees that variations may be due to reduced bodyweight gain in dams but questions that this explains the increase in malformations. Moreover, the incidence of malformations was increased also in mid dose where the body weight gain was similar to controls. Finally, even if this dose level caused mortality in a different study, there were no mortalities among the 15 high dose dams in this study. Therefore, a direct treatment-related effect on foetuses cannot be excluded.

Table 6.6.2.016-5: Incidence of foetal malformations and variations

Dose level (mg/kg bw/day)	**0**	**125**	**250**	**500**
Anterior arch of the atlas poorly ossified	2 (1)	2 (1)	1 (1)	4 (2)
Anterior arch of the atlas split	0 (0)	0 (0)	2 (1)	3 (1)
Extra thoracic centrum and arch	1 (1)	3 (2)	2 (1)	5 (3)
Thoracic centrum only one ossification centre	1 (1)	0 (0)	1 (1)	3 (2)
Thoracic centra fused	2 (1)	1 (1)	1 (1)	2 (1)
Extra ribs on thoracic centra and arch 13 bilateral	1 (1)	0 (0)	3 (2)	5 (4)
Sternebra 6 poorly ossified	2 (1)	1 (1)	2 (1)	4 (2)
Sternebra(e) split	2 (1)	2 (1)	1 (1)	5 (3)
Sternebra(e) unossified	3 (2)	1 (1)	3 (2)	6 (4)
Pubis, poorly ossified	3 (2)	2 (2)	3 (1)	4 (3)
Some ossification in knee area	1 (1)	0 (0)	3 (2)	4 (3)
Skull bones poorly ossified	1 (1)	3 (2)	2 (1)	2 (2)
Frontal, hole in bone	0 (0)	1 (1)	2 (2)	2 (2)
Reduced number of caudal segments	1 (1)	2 (2)	1 (1)	3 (2)

Assessment and conclusion by applicant:
The NOAEL for maternal and developmental toxicity is considered to be 250 mg/kg bw/day based on reduced food consumption and body weight gain at 500 mg/kg bw/day in does. Developmental effects were visible as foetolethality and several malformations (external, visceral, skeletal) at high dose levels: total number of foetuses per litter with malformations was higher in the groups receiving the mid and high dose lever, but without statistical significance. However, it remains unclear, whether statistical analysis of the data had been performed at all. Ventricular septal defects were noted in 2 out of 12 litters in the high dose group (control incidence 0/13 litters). The higher number of further visceral malformations at the top dose level was due to absent kidneys and postcaval lung lobes. Because no individual data are provided it is not identifiable, whether the malformations described were confined to single foetuses or if the foetuses were multiple malformed.

Assessment and conclusion by RMS:
The RMS agrees with the shortcomings listed and with a maternal NOAEL set at 250 mg/kg bw/d based on reduced food consumption and reduced body weight gain. However, in contrast to the applicant and the previous evaluation a developmental NOAEL at 250 mg/kg bw/d is not agreed. Since the incidence of malformations were increased in all treated groups, it is not possible to set a NOAEL for developmental toxicity. Nevertheless, the study has severe deficiencies in reporting and it is therefore difficult to understand data and assess the reliability of results. Among things, it is unclear if aborted foetuses are included in litter data. The number of animals in the study was 15, there were three non-pregnant dams and two with abortions. The number of litters from high dose dams examined is yet stated to be 12.

The RMS does not consider the results of this study reliable.

ED related parameters not investigated: anogenital distance (AGD) in foetuses, indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH). Uterine weight not presented.

B.6.6.2/17

Data point	Data point CA 5.6.2/017 Also presented in 5.6.2/007
Report author	Anonymous
Report year	1981
Report title	Teratological investigation of glyphosate in rats and rabbits
Report No.	Not stated
Document No.	Not stated
Note from the RMS	This study is presented and assessed in B.6.6.2/007 The part of the study investigating rabbit was not included by the applicant.
Acceptability/Reliability	The study is considered non-acceptable

B.6.6.2/18

Data point	CA 5.6.2/018
Report author	[redacted]
Report year	1980
Report title	Technical Glyphosate: Pilot teratology study in rabbits
Report No.	401-055
Document No.	Not reported
Guidelines followed in study	Not applicable (pilot study)
Deviations from current test guideline	Not applicable (pilot study)
Previous evaluation	No
GLP/Officially recognised	No but includes a signed QA statement

testing facilities	
Acceptability/Reliability	Considered "supportive" by the RMS (and by applicant).

MATERIALS AND METHODS

Test material: Technical glyphosate
Lot/Batch: XHJ-64
Purity/Radiochemical purity: 100 %
Vehicle: 1% aqueous Methocel®

Doses of 0, 125, 250, 500, 1250 and 2500 mg/kg bw/day test substance suspended in 1% aqueous Methocel® was administered groups of 5 artificially inseminated female Dutch Belted rabbits by gavage from gestation day 6 to 27. All rabbits were observed once daily for clinical signs of toxicity and mortality once prior to treatment and daily during the treatment and post-treatment period. Individual body weights of dams were recorded on gestation days 0, 6, 12, 18, 24 and 28. All surviving rabbits were sacrificed at scheduled termination on gestation day 28 and the number and location of viable foetuses, early and late resorptions, the number of total implantations and corpora lutea were recorded. The abdominal and thoracic cavities and organs were examined for gross pathological changes. Rabbits that died during the study were necropsied to determine the cause of death. Foetuses were not examined in this pilot study.

RESULTS

One rabbit administered 500 mg/kg bw/day group aborted, the four remaining rabbits in this group and all rabbits administered 1250 and 2500 mg/kg bw/day died. One rabbit at 125 mg/kg bw/day was noted to have a mass in the thoracic area. At necropsy this mass was found to be an abscess and in the absence of similar findings in any other group, a treatment-related effect cannot be excluded.

There were no biologically meaningful differences in appearance or behaviour attributable to treatment with the test item at 125 and 250 mg/kg bw/day when compared to the control.

There were no biologically meaningful differences in mean maternal body weight gains at 125 and 250 mg/kg bw/day when compared to the control. A severs body weight loss was noted at 500 mg/kg bw/day and higher.

Table 6.6.2.018-1: Mortality

Dose level in mg/kg bw/day						
	0	**125**	**250**	**500**	**1250**	**2500**
Spontaneous deaths*	0/5	0/5	0/5	4/5	5/5	5/5
Time of death (gestation day)	--	--	--	15-22	10, 11	9,10
% Mortality	0.0	0	0	80	100	100
Sacrificed after abortion	0	0	0	1**	0	0

* deceased animals / total animals in group
** sacrificed on gestation day 26

The body weight gain was reduced in animals administered doses of 500 mg/kg bw/d and higher but due to the high mortality, only data for animals up not 250 mg/kg bw/d is available for the entire study period.

Table 6.6.2.018-2: Mean body weight development (in g) during gestation

Dose level in mg/kg bw/day						
	0 (Control)	**125**	**250**	**500**	**1250**	**2500**
Animals per	5	5	5	5	5	5

group						
Day of gestation						
0	2808	2646	2793	2760	2791	2596
6	2897	2759	2855	2817	2872	2691
12	2958	2735	2898	2627	--	--
18	3013	2792	2926	2508	--	--
24	3036	2886	2987	2413	--	--
28	3012	2934	2944	--	--	--

At necropsy, yellow foci on the gall bladder were noted in one rabbit administered 500 mg/kg bw/day and in one administered 1250 mg/kg bw/day. Two rabbits administered 500 mg/kg bw/day and one rabbit administered 1250 mg/kg bw/day had erosions of the stomach mucosa. One 1250 mg/kg bw/d rabbit also had erosions of the oesophagus and gall bladder. Varying degrees of autolysis were noted in the other animals dying on study. In one 2500 mg/kg bw/day rabbit, a perforated oesophagus was noted at necropsy and death was thus attributed to an intubation error. A slight increase in the mean number of early and late resorptions and post-implantation loss was noted at 250 mg/kg bw/day but the uterine data is difficult to interpret in a meaningful way since no data is available for the next dose levels due to the high mortality.

Table 6.6.2.018-3: 28–day uterine examination data

Dose level in mg/kg bw/day	**0 (Control)**	**125**	**250**	**500**	**1250**	**2500**
Surviving dams at caesarean section*	5/5	5/5	5/5	0/5	0/5	0/5
Pregnant rabbits	3/5	3/5	5/5	0/5	0/5	0/5
Non-pregnant rabbits	2/5	2/5	0/5	n.a.	n.a.	n.a.
Abortions	0/5	0/5	0/5	1/5	n.a.	n.a.
Viable foetuses - total	13	24	34	0	0	0
Viable foetuses - mean	4.3	8.0	6.8	0.0	0.0	0.0
Non-viable foetuses total	0	0	0	n.a.	n.a	n.a
Late resorptions - total	0	0	1	n.a.	n.a.	n.a.
Early resorptions - total	1	0	2	n.a.	n.a.	n.a.
Early resorptions - mean	0.3	0.0	0.6	n.a.	n.a.	n.a.
Post-implantation loss - total	1	0	3	n.a.	n.a.	n.a.
Post-implantation loss -	0.3	0.0	0.6	n.a.	n.a.	n.a.
Implantations - mean	4.7	8.0	7.4	n.a.	n.a.	n.a.
Corpora lutea	7.0	13.7	12.6	n.a.	n.a.	n.a.

- mean						

* number of surviving animals / total animals in group
n.a. no animals survived until scheduled caesarean section

Assessment and conclusion by applicant:
In the pilot dose range-finding study, the oral administration of glyphosate acid to pregnant rabbits by gavage from gestation gay 6-27 did not result in maternal toxicity at 125 and 250 mg/kg bw/day; there were no treatment-related effects on pregnancy or developmental parameters at these dose levels. At 500 mg/kg bw/day one animal aborted and the four remaining animals died. At 1250 and 2500 mg/kg bw/day all animals died. Accordingly, under the conditions of this study, doses 500 mg/kg bw/day and higher clearly exceeded the maximum tolerated dose (MTD).

Assessment and conclusion by RMS:
The RMS agrees that doses of 500 mg/kg bw/day and higher clearly exceeded the maximum tolerated dose (MTD). The substance seems irritating to the gastrointestinal tract. No further conclusions can be drawn from this pilot study.

B.6.6.2/19

Data point	CA 5.6.2/019
Report author	[redacted] *et al.*
Report year	1980
Report title	Technical Glyphosate: Teratology study in rabbits
Report No.	401-056
Document No.	Not reported
Guidelines followed in study	Not stated. (pre-guideline; satisfies in general the requirements of OECD 414 (1981), but not of OECD 414 (2018))
Deviations from current test guideline	Stated by the applicant: Significant mortality at the higest dose tested yielded less litters than necessary to confirm the observed lack of developmental toxicity at 350 mg/kg bw/day. Additional deviations noted by the RMS: • Clinical data is not included. • Histopathological results not included. • Weight and histopathological changes of the thyroid glands of the dams, uterine weight not presented, anogenital distance (AGD) in foetuses, • Indication of incomplete testicular descent/cryptorchidism in male foetuses, blood samples from dams to assess thyroid hormones (T4, T3 and TSH) were not collected. • Individual foetal data not available • The number of surviving dams was lower than recommended thus the number of litters in treated groups and controls was too low for a robust assessment.
Previous evaluation	Considered “supplementary” in the RAR from 2015.
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	As supportive information

MATERIALS AND METHODS

Test material: Glyphosate Technical
Lot/Batch: XHJ-64
Purity/Radiochemical purity: 98.7 %
Vehicle: 1% aqueous Methocel®

RESULTS

There was a dose-related increase in mortality. For five of the rabbits that died spontaneously, the cause of death was attributed to pneumonia (low dose), respiratory disease (high dose), enteritis or gastroenteritis and caecal ulcerations (high dose). For one rabbit of the mid-dose group and the other 7 rabbits of the high dose group, the cause of death could not be determined.

Table 6.6.2.019-1: Mortality

	(0 mg/kg bw/day)	(75 mg/kg bw/day)	(175 mg/kg bw/day)	(350 mg/kg bw/day)
Spontaneous deaths*	0/16	1/16	2/16	10/17
Time of death (gestation day)	--	26	22, 25	3 to 21
% mortality	0.0	6.3	12.5	58.8
Sacrificed after abortion	2	0	1	1
Sacrificed on gestation day	22	--	27	23

* dead animals / total animals in group

Clinical signs consisting of soft stool and diarrhea were noted in all treated groups during the treatment period. The incidence was slightly increased in mid dose animals but in high dose group either soft stool, diarrhea or both were observed in each animal at least once during the treatment period. Also in the high dose group, there was an increased incidence of animals with nasal discharge.

There were no treatment-related effects on maternal body weights and body weight gain.

Table 6.6.2.019-2: Body weight gain

Dose level in mg/kg bw/day	0 (Control)	75	175	350
Day of gestation				
0	2958 ± 146.6	2876 ± 176.3	2983 ± 157.5	2834 ± 196.9
6	2988 ± 177.5	2937 ± 187.0	3012 ± 206.9	2875 ± 232.1
12	3039 ± 165.6	2986 ± 191.5	3029 ± 216.1	2732 ± 330.5
18	3072 ± 166.4	3002 ± 213.4	2959 ± 276.6	2827 ± 317.2
24	3038 ± 182.3	3005 ± 219.9	2914 ± 321.2	2999 ± 315.1
28	3030 ± 231.7	3008 ± 142.7	2958 ± 307.5	2948 ± 238.7 (↓3 %)

Necropsy data was not included in the original report and there is no information on any changes.

Table 6.6.2.019-3: 28–day uterine examination data

	(0 mg/kg bw/day)	(75 mg/kg bw/day)	(175 mg/kg bw/day)	(350 mg/kg bw/day)
Animals on study	16	16	16	17
Surviving dams at caesarean section*	14/16	15/16	13/16	6/17

Rabbits examined on Day 28	14/16	15/16	13/16	6/17
Pregnant rabbits	12/14	15/15	11/13	6/6
Non-pregnant rabbits	2/14	0/15	2/13	0/6
Abortions	2/16	0/16	1/16	1/17

* number of surviving animals / total animals in group

There were no statistically significant differences in the mean numbers of early or late resorptions, total implantations, corpora lutea, foetal body weights or foetal sex ratio in any of the test substance groups but the low number of high dose dams precludes the possibility of a robust assessment of possible adverse effect on embryofoetal development at this dose level.

The mean foetal body weights were slightly decreased in the test substance groups compared to concurrent controls but the mean foetal body weights in all test substance groups were comparable to the historical control data included (there is no information regarding the origin of the historical control data, e.g. lab, year, strain, experimental conditions etc).

Table 6.6.2.019-4: 28-day uterine examination data

	Historical control (no further information available)	**(0 mg/kg bw/day)**	**(75 mg/kg bw/day)**	**(175 mg/kg bw/day)**	**(350 mg/kg bw/day)**
Pregnant dams#	24	12	15	11	6
Viable foetuses/dam	6.7	5.3 ± 2.73	7.6* ± 1.84	5.9 ± 2.77	6.3 ± 2.25
Post implantation loss/dam##	0.8	0.7 ± 0.89	0.4 ± 0.63	0.2 ± 0.40	0.8 ± 1.33
Total implantations /dam##	7.5	5.9 ± 2.39	8.0 ± 1.81	6.1 ± 2.84	7.2 ± 2.93
Corpora lutea/dam##	10.1	9.0 ± 2.13	10.1 ± 1.64	10.5 ± 3.45	8.5 ± 1.87
Foetal sex distribution (males/females) #	83/77	28/35	53/61	32/33	17/21
Mean foetal body weight (g) ##	30.9	33.4 ± 7.27	30.9 ± 4.43	29.9 ± 7.21	29.3 ± 4.82 (↓5%)

Total number at caesarean section
Number ± SD; historical control without SD
* Statistically significant difference compared to control ($p<0.05$)

The percentages of foetuses with skeletal malformations were higher in treated groups compared to controls but there was no consistency between the types of malformations between treated animals thus effects seems spontaneous rather than related to treatment. There were no visceral malformations observed in any of the dose groups including control and no statistically significant differences in the variation observed or any clear dose-response pattern in the test substance group when compared to the control group (see table below).

Table 6.6.2.019-5: Summary of foetal malformations and variations

	Hist. contr.	**Control**	**75 mg/kg bw/day)**	**175 mg/kg bw/day)**	**350 mg/kg bw/day**
Number of litters examined		12	15	11	6

	%	x/y	%	x/y	%	x/y	%	x/y	%
Skeletal malformations		0/63	0.0	3/114	2.6	2/65	3.1	2/38	5.3
Exencephaly	--	0/63	0.0	0/114	0.0	1/65 (1/11)	1.5	0/38	0.0
Acrania	--	0/63	0.0	0/114	0.0	0/65	0.0	1/38 (1/6)	2.6
Scoliosis with associated rib anomalies	0.6	0/63	0.0	2/114 (2/15)	1.8	0/65	0.0	0/38	0.0
T1 rib absent	--	0/63	0.0	0/114	0.0	1/65 (1/11)	1.5	0/38	0.0
Carpal flexure	0.6	0/63	0.0	0/114	0.0	0/65	0.0	1/38 (1/6)	2.6
Fused cervical vertebral centra	0.6	0/63	0.0	1/114 (1/15)	0.9	0/65	0.0	0/38	0.0
Visceral malformation	--	0/63	0.0	0/114	0.0	0/65	0.0	0/38	0.0
Total malformations		0/63	0.0	3/114	2.6	2/65	3.1	2/38	5.3
Variations									
27 presceral vertebrae	8.7	6/63 (5/12)	9.5	7/114 (3/15)	6.1	9/65 (4/11)	13.8	7/38 (5/6)	18.4
13th rudimentary rib(s)	3.7	5/63 (3/12)	7.9	14/114 (6/15)	12.3	3/65 (3/11)	4.6	3/38 (3/6)	7.9
13th full rib(s)	8.1	3/63 (3/12)	4.8	10/114 (4/15)	8.8	5/65 (2/11)	7.7	6/38 (3/6)	15.8
Hyoid arches bent	--	--	--	2/114 (1/15)	1.8	1/65 (1/11)	1.5	--	--
Hyoid body unossified	--	6/63 (2/12)	9.5	2/114 (2/15)	1.8	6/65 (3/11)	9.2	--	--
Parietals reduced in ossification	0.6	1/63 (1/12)	1.6	--	--	1/65 (1/11)	1.5	--	--
Sternebrae #5 and/or #6 unossified	5.6	6/63 (3/12)	9.5	13/114 (7/15)	11.4	13/65 (5/11)	20.0	4/38 (2/6)	10.5
Pubis unossified	--	4/63 (1/12)	6.3	1/114 (1/15)	0.9	4/65 (1/11)	6.2	--	--
Talus unossified	--	3/63 (1/12)	4.8	--	--	5/65 (3/11)	7.7	--	--
Extra ossification center, cervical area	--	--	--	--	--	1/65 (1/11)	1.5	--	--
Major vessel variations	8.7	11/63 (6/12)	17.5	14/114 (8/15)	12.3	14/65 (5/11)	21.5	6/38 (4/6)	15.8

x/y number of foetuses affected / total number of foetuses examined
(a/b) number of litters affected / total number of litters

Assessment and conclusion by applicant:
The study was performed before GLP and the respective OECD test guidelines were established. Nevertheless, the study is generally in concordance with the current OECD 414 with the restriction that the group sizes are smaller than requested in this guideline. Therefore, the outcome can be reported as valid. The oral administration of glyphosate acid to time-mated rabbits by gavage from gestation day 6 to 27 resulted in

maternal toxicity at ≥ 175 mg/kg bw/day. There were no treatment-related effects on pregnancy or foetuses at any dose level that could not be attributed to maternal toxicity. Therefore, the NOAEL was considered to be 75 mg/kg bw/day for maternal toxicity. The NOAEL for developmental toxicity was considered to be 175 mg/kg bw/day because of the high dose offspring NOAEL of 350 mg/kg bw/day could not be relied upon because maternal mortality resulted in only six litters for six surviving dams.

Assessment and conclusion by RMS:

Since the mortality rates in the intermediate- and the high-dose groups were above 10 %, doses ≤ 175 mg/kg bw/day were apparently too high. According to OECD 414 *"Maternal mortality greater than 10 % is considered excessive and the data for that dose level shall not normally be considered for further evaluation."*

The RMS agrees with a maternal NOAEL of 75 mg/kg bw/d based on mortality observed at higher dose levels. Furthermore, due to the high mortality, less than 16 dams/group is available for the mid and high dose groups and thus below the number recommended in OECD TG 414 (16). The number of foetuses available for examinations was 63, 114, 65 and 38 in control, low, mid and high dose respectively. Even though the number of litters was similar between the mid dose and the control group, it is not considered possible to exclude that the lower number of foetuses in treated animals could mask treatment-related effects occurring at a low incidence thus the RMS considers the only reliable developmental NOAEL to be at or above 75 mg/kg bw/day in this study. This is lower than the developmental NOAEL set at 175 mg/kg bw/d in the previous evaluation. However, it should be noted that this NOAEL is less reliable since there were too few dams and thus too few litters available for the assessment.

B.6.6.3. Reproductive toxicity - Information from public literature

A literature search for the active substance glyphosate was performed by the applicant in accordance to the provisions of the EFSA Guidance "Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) 1107/2009" and updated Appendix to this Guidance document. The articles considered relevant were categorized by the applicant as A (providing data for establishing or refining risk assessment parameters), as B (articles relevant to the data requirement but in opinion of the applicant only to provide supplementary information that does not alter existing risk assessment) or as C (articles of unclear relevance).

B.6.6.3.1. Public literature relevant for section B.6.6.1

Summary of published literature studies identified by the applicant as relevant and reliable or reliable with restrictions (Category A):

Category A – Gorga, A. *et al.*

Data point	EU data requirement No. CA 5.6.1/015
Report author	Gorga, A. *et al.*
Report year	2020
Report title	In vitro effects of glyphosate and Roundup on Sertoli cell physiology
Document No.	Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes /Reliable with restrictions

Aim of the study
The aim of the study was to analyse whether glyphosate and/or glyphosate-based formulation (Roundup) can affect Sertoli cell functions, such as energy metabolism and blood-testis barrier integrity, which are essential to maintain spermatogenesis.

Materials and methods

Test substance	Glyphosate purchased from Sigma-Aldrich (St Louis, MO, USA) and Glyphosate formulation Roundup Full II (Monsanto Argentina S.A.I.C.) containing 54% w/v acid glyphosate.
Lot/Batch	Not specified
Purity/Radiochemical purity	Not specified
Sertoli cell (SC) isolation and culture	Twenty–day-old Sprague-Dawley rats (Rattus norvegicus) were obtained from the Animal Care Laboratory, Facultad de Ciencias Veterinarias, Buenos Aires, Argentina. Animals were killed by CO_2 asphyxiation according to protocols for animal laboratory use following the principles and procedures outlined in the National Institute of Health Guide for Care and Use of Laboratory Animals. The protocol was approved by the Comité Institucional de Cuidado y Uso de Animales de Laboratorio (CICUAL) from the Hospital de Niños "Dr. Ricardo Gutiérrez". SC were isolated as previously described. Decapsulated testes were digested with 0.1 % w/v collagenase and 0.006 % w/v soybean trypsin inhibitor in Hanks' balanced salt solution (HBSS) for 5 min at room temperature by manual agitation. The enzymatic action was stopped by dilution with four volumes of HBSS. Seminiferous tubules were collected by sedimentation and washed twice with HBSS. Then seminiferous tubules were cut with a razor and submitted to 1M glycine-

	2mM EDTA (pH 7.4) treatment for 10 min to remove peritubular cells. At the end of the incubation period, nine volumes of HBSS were added and a 30 min sedimentation was performed. The washed tubular pellet was then digested again with 0.1 % w/v collagenase and 0.006 % w/v soybean trypsin inhibitor in HBSS for 10 min at room temperature by continuous pipetting. The enzymatic action was stopped by dilution with four volumes of HBSS. The cell suspension was collected by centrifugation at 200 x g for 3 min. The cell suspension was diluted with HBSS and submitted to a 10 min sedimentation to remove germ cells. The pellet containing SC was filtered through a nylon mesh and SC were recovered by centrifugation at 200 x g for 3 min. SC were resuspended in culture medium which consisted of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, supplemented with 10mM HEPES, 100 IU/mL penicillin, 2.5 µg/mL amphotericin B, 1.2 mg/mL sodium bicarbonate, 10 µg/mL transferrin, 5 µg/mL insulin, 5 µg/mL vitamin E and 4 ng/mL hydrocortisone. SC were cultured on 6-, 24- or 96-multiwell plates (5 µg DNA/cm^2), on Matrigel-coated cell culture inserts (15 µg DNA/cm^2) placed on 24-multiwell plates or on glass coverslips coated with laminin at 34 °C in a mixture of 5 % CO_2:95 % v/v air. No myoid cell contamination was revealed in the cultures when an immunoperoxidase technique was applied to SC cultures using a specific antiserum to smooth muscle α actin. Remaining cell contaminants were of germ cell origin and this contamination was below 5 % after 48 h in culture as examined by phase contrast microscopy.
Culture conditions	SC were allowed to attach for 48 h in the presence of insulin and medium was replaced at this time with fresh medium without insulin. Treatment with glyphosate (G) and Roundup (R) was performed with variable doses and for variable periods of time. Cells incubated for 48 h with 10, 100 and 1000 ppm of G and R harvested on day five were used to evaluate cell viability and LDH leakage. The cells treated for 48 h with 100 ppm of G and R were harvested on day five and used to evaluate GLUT1, FAT/CD36 and CPT1 mRNA levels, glucose uptake and fatty acid (FA) oxidation and the 48 h-conditioned media were utilized to evaluate lactate production. For western blot studies, cells cultured for 4 days under basal conditions and pretreated for 30 min with 10 and 100 ppm of G or R were used. To quantify Transepithelial Electrical Resistance (TER), SC were cultured at high cell density (15 µg DNA/cm^2, corresponding to 1.2×10^6 cells/cm^2) on Matrigel-coated (1,6 dilution with F12/DMEM v/v) cell culture inserts (Millicell HA inserts) (Millipore, Billerica, MA, USA) placed on 24-multiwell plates. On day 3 in culture testosterone was added and TER across SC monolayer was recorded every 24 h in culture. On day 5, when the tight junction barrier had been formed, different doses of G or R were added and TER was recorded until day 8. To study the distribution and localization of claudin11, the cells were cultured on glass coverslips coated with laminin and treated with 100 ppm G or R in the presence or absence of testosterone for 48 h and harvested on day 5.
Evaluation of Sertoli cells	Energetic metabolism in SC has been considered to have features of its own. Lactate, produced by SC, provides the energetic substrate to germ cells in the adluminal compartment. Consequently, it has been postulated that SC utilizes FA as their energy source. In this context, lactate production, glucose uptake, FA oxidation and the expression of genes involved in these processes were evaluated. <u>Lactate determination:</u> Conditioned media obtained from cells cultured in 24-multiwell plates were used to determine lactate production. Lactate was measured by a standard method involving conversion of NAD+ to NADH. <u>Measurement of 2-deoxyglucose (2-DOG) uptake:</u> Glucose transport was studied using the uptake of the labelled non-metabolizable glucose analogue 2-DOG on cells cultured in 24-multiwell plates. <u>Fatty acid oxidation assay:</u> FA oxidation was performed measuring the release of ^3H_2O to the incubation medium from [^{3}H]-palmitate on SC cultured in 24-multiwell.
Evaluation of blood-testis barrier (BTB) function	The main component of the BTB is the presence of tight junctions between neighboring SC. In order to evaluate BTB function, Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly were evaluated.

	Transepithelial Electrical Resistance (TER) measurement: The establishment of the SC junction barrier was assessed daily from day 3 to day 8 by measurement of TER across the SC monolayer by a Millicell electrical resistance system (Millipore). Briefly, a short (~2 s) 20-µA pulse of current was passed through the epithelial monolayer between 2 silver-silver chloride electrodes and electrical resistance was measured. Electrical resistance was then multiplied by the surface area of the insert to yield the area of resistance in ohms.cm^2. The net value of electrical resistance was then computed by subtracting the background, which was determined by Matrigel-coated cell-free inserts. Each time point had quadruplicate bicameral units. This experiment was run four times on different batches of cells. Immunofluorescent (IF) detection of claudin11 protein: Monolayers were fixed with methanol for 10 min at -20 °C. After washes with PBS, cells were permeabilised with 0.1 % Triton X-100 in PBS for 30 min at room temperature. After 3 washes with PBS for 1 min each, the cells were blocked with 5 % bovine serum albumin (BSA). Then, the coverslips were incubated with a 1:50 dilution of polyclonal antibody against claudin11 in PBS overnight at 4 °C. After 3 washes with PBS for 1 min each, coverslips were incubated with an anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated (1,25; Vector Laboratories, Burlingame, CA, USA). For negative controls, primary antibodies were replaced by PBS. Finally, the coverslips were washed 3 times with PBS for 1 min each, mounted in buffered glycerine and observed using an Axiophot fluorescent microscope with epi-illumination (Carl Zeiss Inc., Oberkochen, Germany).
RT-Real-time PCR (RT-qPCR)	The expression of genes that participate in energetic metabolism (GLUT1, FAT/CD36 and CPT1) and in BTB organisation (occludin, claudin11 and ZO-1) was evaluated by RT-qPCR. Total RNA was isolated from SC cultured in 6-multiwell plates with TRI Reagent (Sigma-Aldrich). The amount of RNA was estimated by spectrophotometry at 260 nm. Amplification was carried out as recommended by the manufacturer: 25 µL reaction mixture containing 12.5 µL of SYBR Green PCR Master mix (Applied Biosystems), the appropriate primer concentration and 1 µL of cDNA. The relative cDNA concentrations were established by a standard curve using sequential dilutions of a cDNA sample. The data were normalised to HPRT1. The amplification program included the initial denaturation step at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products. The comparative ΔΔCt method was used to calculate relative gene expression.
Western blot analysis	Cells cultured in 6-multiwell plates were washed once with PBS at room temperature. Then, 200 µL of PBS containing 2 µL of protease inhibitor cocktail (P-8340; Sigma-Aldrich), 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 50 nM okadaic acid and 2 mM PMSF was added to each well. Cells were then placed on ice and disrupted by ultrasonic irradiation. Western blot analysis was then performed. Membranes were probed with antibodies that allow specific recognition of total Akt and mTOR, phosphorylated p38-MAPK and ERK1/2 (Cell Signaling Technology, Inc., Danvers, MA, USA), claudin11 (Zymed Lab. Inc.), androgen receptor and GAPDH (Santa Cruz Biotechnology, Inc., USA). A 1:1000 dilution of primary antibodies, as indicated by the manufacturer, was used. For chemiluminescent detection of the blots, a commercial kit from Cell Signaling Technology was used. The intensities of the autoradiographic bands were estimated by densitometry scanning using NIH Image Software (Scion Corporation). Levels of the corresponding total Akt, mTOR and GAPDH served as loading controls.
Cytotoxicity	A cell viability test (MTT assay) was performed in cells cultured in 96-multiwell using a commercial kit (CellTiter 96® AQueous NonRadioactive Cell Proliferation Assay; Promega Corporation). Cell cytotoxicity was determined by measuring the activity of LDH enzyme leaked from the cytosol of damaged cells into the medium. Results were expressed as the percentage of activity detected in the media over the sum of the activities in the media and in cells.
Statistical analysis	All experiments were run in triplicates and repeated 3–4 times. One way ANOVA and post hoc analysis using Tukey-Krämer's multiple comparisons test were performed using

	InfoStat versión 2016 (Grupo InfoStat, FCA, UNC, Argentina). P values < 0.05 were considered statistically significant.

Results

Effects of glyphosate and Roundup on SC cell viability - SC cultures were exposed for 48 h to glyphosate (G) and Roundup (R) at concentrations ranging from 10 to 1000 ppm, corresponding to 0.01 to 1 g/L respectively. Cell viability was analysed by MTT assay and by measuring LDH leaked from the cytosol of damaged cells into the medium. The highest dose tested for R (1000 ppm) caused a cell death (Fig. 1). Therefore, in the present investigation destined to analyse G and R effects on SC functions only doses of 10 and 100 ppm were utilised.

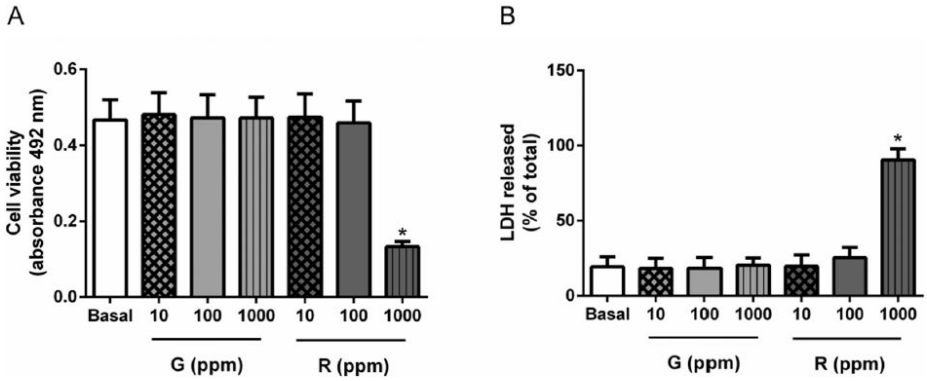

Fig. 1. Effect of G and R on SC cytotoxicity.
SC were maintained under Basal conditions or incubated with 10, 100 or 1000 ppm of G or R for 48 h. (A) Cell viability was determined by MTT assay. (B) LDH activity was determined in SC monolayer and in the culture medium. Values represent mean ± S.D. of one representative experiment out of three. *p < 0.05 versus Basal.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Effects of glyphosate and Roundup on SC energetic metabolism - SC cultures were exposed for 48 h to 100 ppm of G and R. Fig. 2 shows the results obtained for lactate production, glucose uptake and GLUT1 mRNA levels. The exposure to G or R did not modify lactate production neither glucose uptake nor GLUT1 expression. On the other hand, Fig. 3 shows the results obtained for FA oxidation, FAT/CD36 and CPT1 mRNA levels. Again, no variations in the parameters analyzed were observed after G or R exposure.

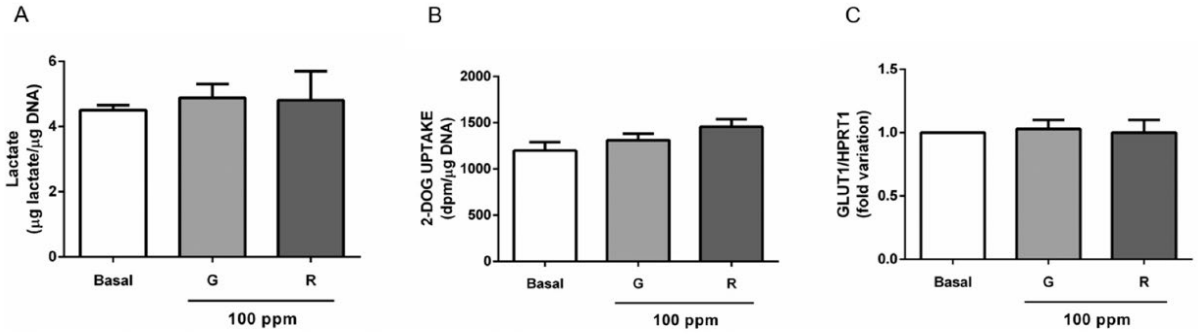

Fig. 2. Effect of G and R on lactate production, glucose uptake and on *Glut1* mRNA levels in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Lactate levels were determined in the conditioned media. (B) Glucose uptake assay (2-DOG uptake) was performed after the 48 h incubation period. (C) Total RNA was extracted and RT-qPCR was performed to detect *Glut1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

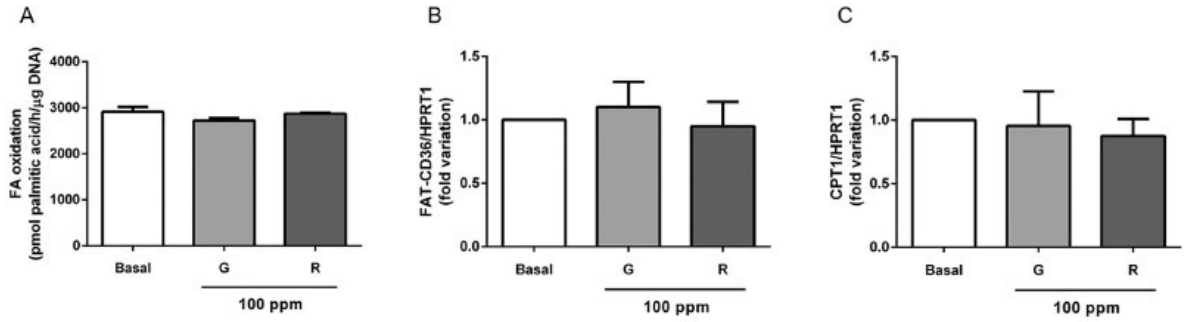

Fig. 3. Effect of G and R on FA oxidation, *FAT/CD36* and *CPT1* mRNA levels in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. (A) Fatty acid oxidation was assessed by measuring ^3H_2O produced in the incubation medium. (B and C) Total RNA was extracted and RT-qPCR was performed to detect *FAT/CD36* and *CPT1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Effects of glyphosate and Roundup on blood-testis barrier integrity - SC supply germ cells with a microenvironment preserved by the BTB. The main component of the BTB is the presence of tight junctions between neighboring SC. The establishment of these junctions between SC in culture was assessed daily from day 3 to day 8 by measuring TER across the SC monolayer. When SC were plated, junctions begin to assemble and an increase in TER was observed. On day 5, when SC completed barrier assembly, G or R were added to the culture medium. Compared with Control (92.3±3.1), a significant decline in TER was produced by the addition of 100 ppm of G (58.0±1.5) or 10 and 100 ppm of R (67.8±2.8 and 62.2±4.9, respectively) (Fig. 4 and Table 2).

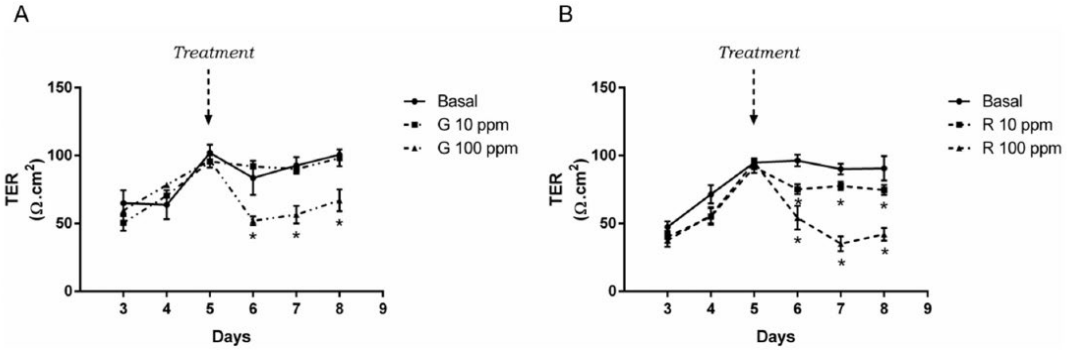

Fig. 4. Effect of G and R on TER across SC.
SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G (A) or R (B) on day 5. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of four. Asterisks indicate significant differences from basal cultures for each particular day, $p < 0.05$.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Table 2
Effect of 48-h treatment with G and R on TER.

	TER ($\Omega \cdot cm^2$)
Control	92,3 ± 3,1
G 10 ppm	85,5 ± 4,5
G 100 ppm	58,0 ± 1,5*
R 10 ppm	67,8 ± 2,8*
R 100 ppm	62,2 ± 4,9*

SC monolayers were maintained under Basal conditions or treated with 10 or 100 ppm of G or R on day 5 for 48 h. Results are presented as means ± SD of four independent experiments (*$p < 0.05$).

(Table adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Considering that it had been demonstrated that p38-MAPK and ERK1/2 signaling pathways were involved in the disruption of BTB integrity by xenobiotics, the possible alteration of these pathways by G or R treatment was evaluated. R increased P-p38-MAPK and P-ERK1/2 levels (Fig. 5B). G did not modify P-p38-MAPK and P-ERK1/2 levels at any dose tested (Fig. 5A). 100 ppm G or R treatment did not modify claudin11, neither occludin nor ZO-1 mRNA levels (Fig. 6). Claudin11 protein levels were not modified by G or R treatment (Fig. 7A). In control conditions, claudin11 was detected at the zone of contact between adjacent cells, in a linear and continuous pattern that delineated cell borders in basal conditions. Addition of 100 ppm G or R induced redistribution of claudin11 since immunofluorescence became discontinuous and was redistributed from the cell surface into the cytoplasm (Fig. 7B).

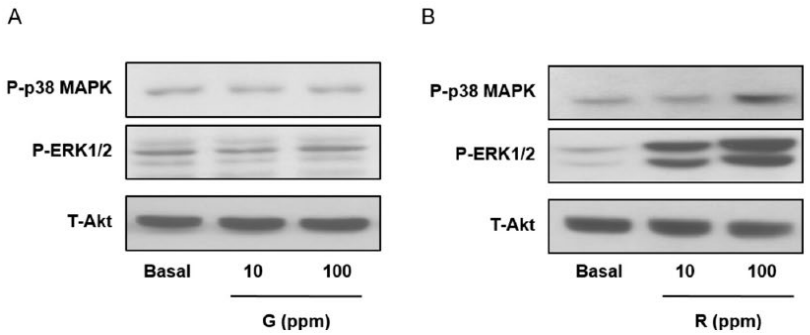

Fig. 5. Effect of G and R on P-p38-MAPK and P-ERK1/2 levels in SC.
SC were maintained under Basal conditions or incubated with 10 or 100 ppm of G (A) or R (B) for 30 min. Western blot analysis was performed utilizing antibodies for phosphorylated p38-MAPK (P-p38-MAPK) and ERK1/2 (P-ERK1/2) or total Akt (T-Akt). Results are representative of 3 independent experiments performed/treatment group.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

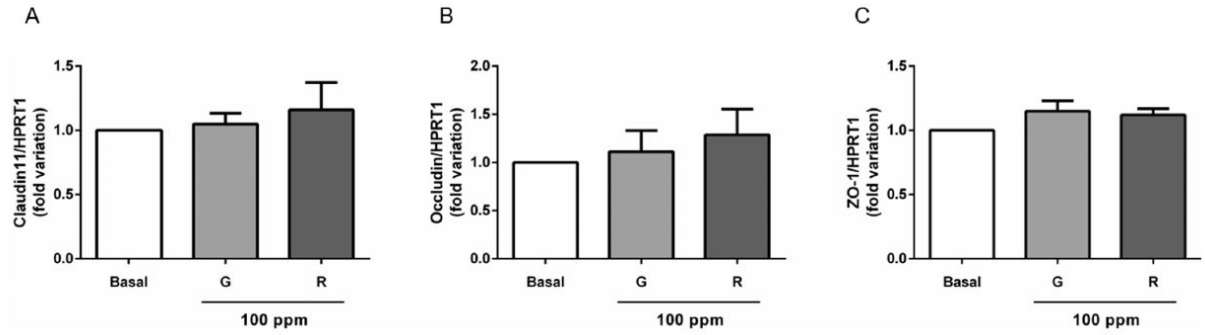

Fig. 6. Effect of G and R on *claudin11, occludin* and *ZO-1* mRNA levels in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. Total RNA was extracted and RT-qPCR was performed to detect *occludin*, *claudin11* and *ZO-1* mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

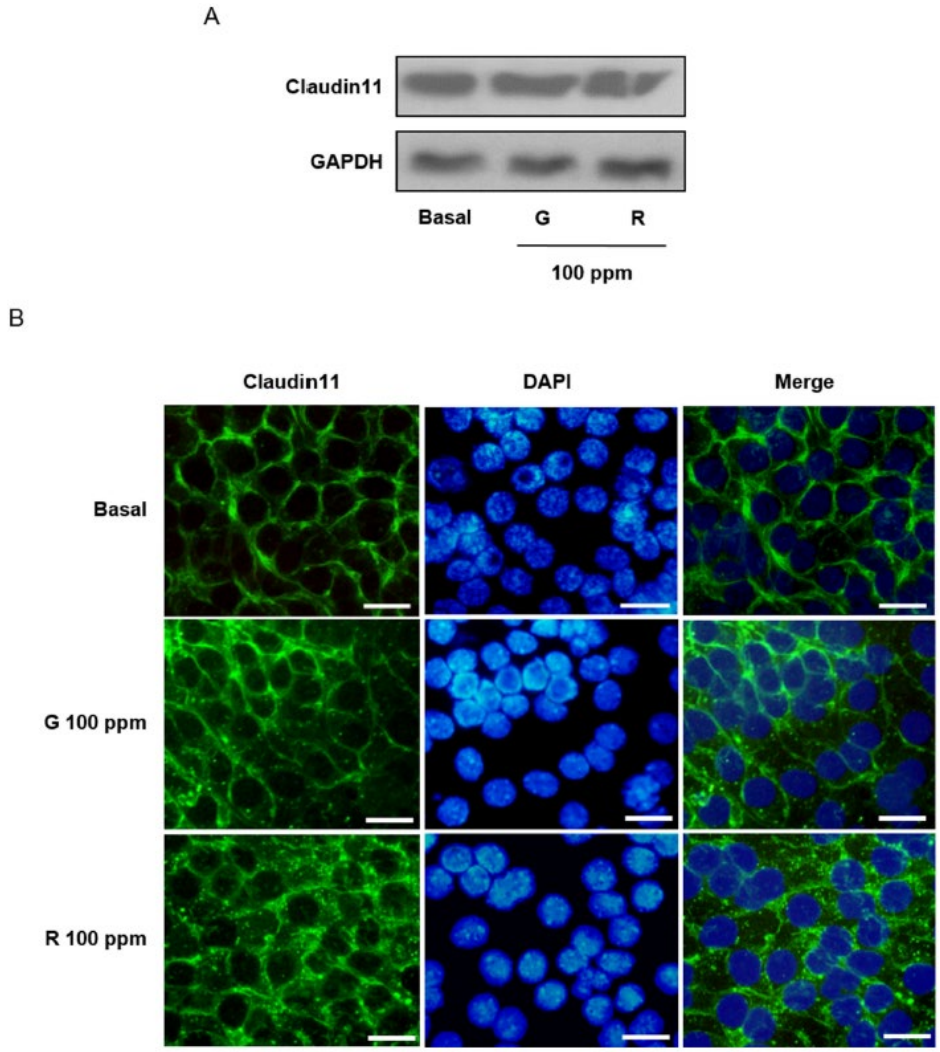

Fig. 7. Effect of G and R on claudin11 protein levels and localization in SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Western blot analysis was performed utilizing antibodies for claudin11 or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. B) Claudin11 was revealed by IF. Bars: 50 µm.
(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Effects of glyphosate and Roundup on testosterone regulation of blood-testis barrier integrity - It is well known that testosterone is the main regulator of BTB function, and that G or R can act as endocrine disruptors. In order to elucidate a possible mechanism responsible for adverse effects of G or R, we decided to evaluate whether herbicides can interfere with androgen action in BTB. Fig. 8A and B shows that 100 ppm G or R treatment did not modify androgen receptor mRNA or protein levels. Fig. 8C and Table 3 shows that testosterone increased TER and that the addition of 100 ppm G or R provoked a significant decline in testosterone stimulated TER values. Fig. 9 shows that, similar to what was observed under basal conditions, 100 ppm G or R treatment induced a redistribution of claudin11 from cell membrane to cytoplasm under the presence of testosterone.

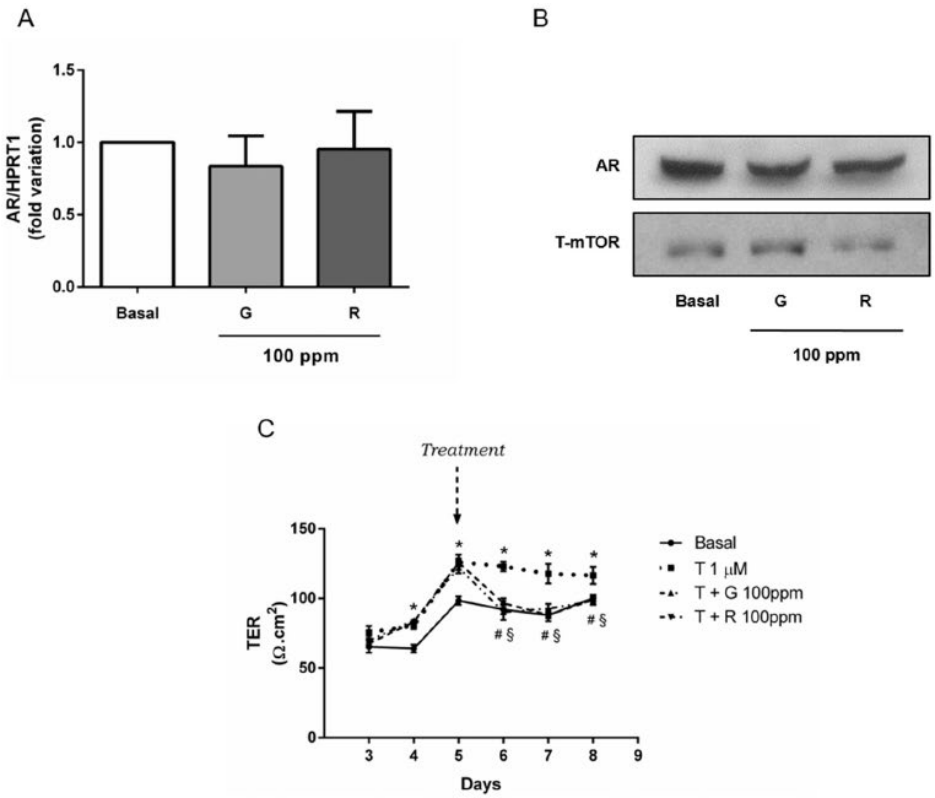

Fig. 8. Effect of G and R on androgen receptor (AR) expression and testosterone regulation of TER across SC.
SC were maintained under Basal conditions or incubated with 100 ppm of G or R for 48 h. A) Total RNA was extracted and RT-qPCR was performed to detect AR mRNA levels. The comparative ΔΔCt method was used to calculate relative gene expression. Graphics show pooled data from four independent experiments performed indicating fold variation in mRNA levels relative to Basal. B) Western blot analysis was performed utilizing antibodies for AR or total Akt (T-Akt). Results are representative of three independent experiments performed/treatment group. C) SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R. TER across SC monolayer was measured from day 3 to 8. Values represent mean ± S.D. of triplicate wells from the same culture of one representative experiment out of three. Symbols indicate significant differences for each particular day: *p < 0.05 T vs Basal; #p < 0.05 T vs T + G; §p < 0.05 T vs T + R.

(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Table 3
Effect of 48-h treatment with G and R on testosterone regulation of TER across SC.

	TER ($\Omega \cdot cm^2$)
Basal	94,7 ± 8,4
T 1 µM	144,9 ± 13,2*
T + G 100 ppm	111,5 ± 12,5
T + R 100 ppm	98,0 ± 6,0

SC monolayers were maintained under Basal conditions or stimulated with testosterone (T) since day 3. On day 5, SC monolayers were treated with 100 ppm of G or R for 48 h. Results are presented as means ± SD of three independent experiments (*$p < 0.05$).

(Table adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

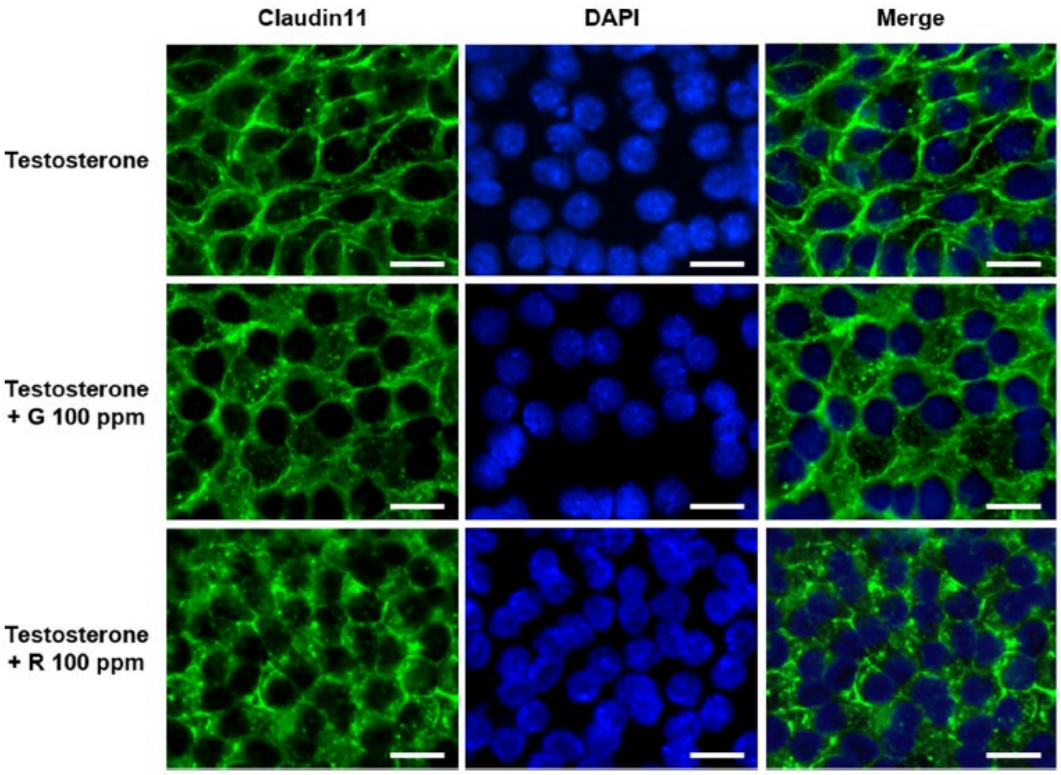

Fig. 9. Effect of G and R on claudin11 localization in the presence of testosterone in SC.
SC were incubated with 100 ppm of G or R in the presence of testosterone for 48 h. Claudin11 was revealed by IF. Bars: 50 µm.
(Figure adapted from Gorga *et al.* (2020). In vitro effects of glyphosate and Roundup on Sertoli cell physiology Toxicology in Vitro, Volume 62, February 2020, 104682 doi.org/10.1016/j.tiv.2019.104682)

Conclusion

In summary, this investigation shows that G and R alter the Sertoli cell junction barrier permeability. This study also shows that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassemble of the barrier. We postulate that BTB integrity is a sensitive target for the adverse effects of G or R on male reproductive function.

Assessment and conclusion

Assessment and conclusion by applicant:

This *in vitro* investigation showed that exposure to G and R at 100 ppm alters Sertoli cell (SC) junction barrier permeability, measured by decreased TER, and also decreased testosterone-stimulated TER. This study also showed that, at least in part, the loss of location of claudin11 at the interface between neighboring Sertoli cells might be responsible for the disassembly of the barrier. G or R did not modify androgen receptor mRNA or protein levels, nor did G modify P-p38-MAPK and P-ERK1/2 signalling pathways involved with BTB integrity at any doses tested, or affect the expression of intercellular junction proteins (claudin11, occludin and ZO-1). However, G and R induced redistribution of claudin11 at the zone of contact between cells. Neither G nor R modified lactate production, glucose uptake, GLUT1, FA oxidation, or FAT/CD36 and CPT1 expression in SC, thus indicating no effect of G or R on SC nutritional function or metabolism.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterised, there was no positive control and most of the endpoints were tested at only 2 concentrations preventing any dose-response evaluations, with the highest concentration exceeding a physiologically relevant dose.

Reliability criteria made by the applicant

Publication: Gorga *et al*., 2020	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup Full II (Monsanto, Argentina)
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described		
Test concentrations in physiologically acceptable range (< 1 mM)	Y	2 test concentrations used of which the highest was > 1 mM (1000 µg/mL).
Cytotoxicity tests reported	Y	
Positive and negative controls	N	No positive control reported.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported		Explored but not found (only 2 concentration levels tested for most endpoints)
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used was not sufficiently characterised and most of the endpoints were tested at only 2 concentrations.		

Assessment and conclusion by RMS:

This study shows that after 48 hour exposure of Sertoli cell (SC) culture (from 20 day old Sprague-Dawley rats) to 100 ppm of glyphosate (G) and glyphosate-based formulation Roundup (R) (containing 54% w/v acid glyphosate) did not modify lactate production, glucose uptake, GLUT1 expression, fatty acid oxidation, FAT/CD36 and CPT1 mRNA levels indicating no effects on Sertoli cell metabolism.

This study also evaluated the effects on G and R on blood-testis barrier (BTB) function (by measuring Transepithelial Electrical Resistance (TER), claudin11 cellular distribution and the expression of proteins that participate in tight junction assembly (claudin11, occludin and ZO-1) and on testosterone regulation of BTB integrity. 100 ppm of G and 10 and 100 ppm of R produced a significant decline in TER after 24 and 48 hour treatment. While R increased P-p38-MAPK and PERK1/2 levels, G did not affect these indicating that G and R exposure affected TER levels independent of p38-MAPK and ERK1/2 pathways. Also 100 ppm of G and R did not affect claudin11, occludin or ZO-1 mRNA levels. However, even though claudin11 protein levels were not affected, 100 ppm G and R induced redistribution of claudin11 from the cell surface into the cytoplasm. 100 ppm G and R did not affect androgen receptor mRNA or protein levels in BTB but significantly decreased testosterone-stimulated TER values.

In conclusion, under the conditions of the study, G and R altered the Sertoli cell junction barrier permeability which could partly be due to redistribution of claudin11 from the cell surface into the cytoplasm.

The study is relevant for risk assessment as it informs on the effects at cellular level that could lead to adverse effects on male reproductive function. The conclusions from the study should be used in a weight of evidence assessment. The study is reliable with restrictions as the test material identity, in particular purity, is not specified; only 1 or 2 concentration levels were tested; and a positive control was missing.

Category A – Manservisi, F. *et al.*

Data point	CA 5.6.1/016
Report author	Manservisi, F. *et al.*
Report year	2019
Report title	The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system
Document No.	Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

The aim of the (pilot) study was to examine whether exposure to glyphosate-based herbicides (GBHs) at a dose of glyphosate considered to be "safe" (the US Acceptable Daily Intake of 1.75 mg/kg bw/day), starting from in utero life, affect the development and endocrine system across different life stages in Sprague-Dawley rats.

Materials and methods

Test substance and its purity	Glyphosate (purity of > 99.5 %), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy). Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5 %), water (42.5 %) and surfactant (16 %)) was purchased from Consorzio Agrario dell'Emilia, Bologna, Italy
Lot/Batch	Not specified
Animals and experimental design	Each of the 24 virgin female Sprague-Dawley (SD) rats (17 weeks old, 270–315 g) was mated outbred with one breeder male rat of the same age and strain. Every day, the females were examined for the presence of sperm. After evidence of mating, females were housed separately during gestation and delivery. Newborns were housed with their mothers until weaning. Weaned offspring were co-housed, by sex and treatment group, not more than 3 per cage. Cages were identified by a card indicating study protocol code, experimental and pedigree numbers, and dosage group. The cages were placed inside a single room at 22 °C ± 3 °C and at 50 ± 20 % relative humidity. A light/dark cycle of 12 hours was maintained. Two groups of dams and their pups were treated with either glyphosate or MON 52276 diluted in drinking water to achieve the desired glyphosate dose of 1.75 mg/kg bw/day. The F0 female breeders received the test item from gestation day (GD) 6 to the end of lactation, while the offspring (F1) continued to be exposed after weaning for an additional 6 or 13 weeks. Glyphosate or MON 52276 solutions were freshly prepared on a daily basis taking into account body weight and water consumption. During pregnancy and lactation, embryos and offspring (F1) were all retained in the litter and received the test compounds mainly through their dams (F0). The day of birth was designated post-natal day 1 (PND 1) for pups and lactation day 1 (LD 1) for dams. After weaning, the offspring (F1) were treated via the drinking water until sacrifice. On PND 28, offspring were randomly distributed in two

	cohorts: 8/sex/group of the 6–week cohort and 10/sex/group of the 13–week cohort. Altogether, 108 rats (54 males and 54 females) were enrolled in the post-weaning treatment phase.
Measurements in F0 dams and litters prior to weaning	Mean gestational length was calculated as the number of days from detection of a positive vaginal smear (GD 0) to birth of a litter. Pregnancy was confirmed by the occurrence of parturition. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 (last measurement before weaning). Body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. Feed and water consumption of the dams was recorded twice weekly during gestation on GD 0, 3, 6, 9, 12, 15, 18, and 21, and twice weekly during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21 and 25. Dead pups were removed when found and sexed when possible. Sex was determined on PND 1. The mean litter size was assessed on PND 0 (within 24 hours from delivery), 1, 4, 7, 10, 13, 16, 19, 21, and 25. Litter size included dead as well as live offspring. Dead pups were visually examined by floating the lungs in saline, to distinguish if they were stillborn (died in utero) or died shortly after birth. Live-birth index was determined on PND 0 as (number of pups born alive / total number of pups born) × 100. Survival index, calculated as (total number of live pups at designated time point / number of live pups born) × 100, was measured on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. For all the pups, anogenital distance (AGD) was measured and body weight recorded on PND 4.
Post weaning endpoints up to adulthood	After weaning, body weight was measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice, the means of individual body weights were calculated for each group and sex. Daily water and feed consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. Time to vaginal opening (VO) was determined by daily inspection of all female pups starting on PND 28. The body weight of each female was recorded on the day VO was observed. Time to balano-preputial separation (BPS) was determined by daily inspection of all males beginning on PND 35. The body weight of each male was recorded on the day BPS was observed. The female rats belonging to the developmental (6–week) cohort were also monitored for the time to first estrous (FE).
Estrous cycle characterisation	Starting on approximately PND 95 and for the duration of 3 weeks, daily vaginal lavage was performed on female rats of the 13–week cohort. To reduce variability, vaginal cytology samples were collected by vaginal lavage at the same time of the day over the course of the experiment, in the mid-morning, between 10:00 and 13:00 h. Collection, processing and vaginal smear evaluation was performed as described previously in Eur J Oncol. 2018;23(2):80–5.
Necropsy	Five days after weaning, dams were sacrificed and following tissues were collected and fixed in alcohol: mammary glands (4 sites: axillary and inguinal, right and left), adrenal glands, uterus (including cervix), ovaries, and vagina. The adrenal glands, uterus and ovaries were also weighed. For the determination of serum testosterone, blood was collected and serum prepared by centrifugation and stored at -80 °C pending analysis. All male and female pups belonging to both cohorts were sacrificed on PND 73 ± 2 for the 6–week cohort and PND 125 ± 2 for the 13–week cohort. Following tissues were collected for histopathology and fixed in alcohol: mammary gland (4 sites: axillary and inguinal, right and left), thyroid and parathyroid, adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left and right testis with epididymis (half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation), uterus (including cervix), ovaries and vagina. During necropsy, all tissues with gross lesions were removed for histopathology. Adrenal glands, bladder and prostate, seminal vesicles and coagulating gland, left testis, left epididymis, uterus (including cervix) and ovaries were weighed. In case of paired organs, both organs were preserved. Organ weight was expressed as absolute and relative organ weight. Rats were sacrificed randomly across the 4 stages of the estrous cycle. In order to determine and allow correlation with histopathology in reproductive organs and hormone analysis, the stage of estrous cycle was determined by histological appearance of the various components of the reproductive tract for F1 females of the 6–week cohort or by a vaginal smear examined on the day of necropsy for F1 females of the 13–week cohort.
Sperm analysis	Sperm analyses were performed on each male from both cohorts, at scheduled necropsies on PND 73± 2 and PND 125 ± 2. At necropsy, half of the right testis and the whole right epididymis were frozen in liquid nitrogen and stored at -80 °C until evaluation. Spermatids

	resistant to homogenisation and spermatozoa present in the caput/corpus and cauda epididymis were counted. The tunica albuginea was removed from the (half) testicle, and a sample of the parenchyma was weighed and homogenised in 5 mL saline-TritonX-100 at 0.05 %. The samples were then diluted 10-20 times in saline, and the mature spermatids resistant to homogenisation (step 17-19 spermatids) were counted using a Thoma chamber. Four fields per animal were recorded, and the numbers of spermatids per gram of testis were calculated. To calculate the daily sperm production (DSP) these values were subsequently divided by 6.1, which is the number of days step 17-19 spermatids are present in the seminiferous epithelium. Similarly, the segments of the epididymis (caput, corpus and cauda) were cut with a scissor, weighed, homogenised, diluted and counted as described for the testes. The number of spermatozoa in each homogenate was determined and the total number of spermatozoa for each segment of the epididymis calculated. The epididymal sperm transit time through the epididymal caput/corpus and cauda was calculated by dividing the number of spermatozoa present in each portion of the epididymis by the DSP of the associated testis. To assess the percentage of morphologically abnormal sperm half of left cauda epididymis of each rat was transferred to a Petri dish containing 2.5 mL (for 70 day old animals) or 3.5 mL (for 120 day old animals) of Dulbecco's PBS at 37 °C, cut in 2-3 pieces and incubated for approximately 3 minutes at 37 °C with gently swirling to facilitate release of sperm cells from the cauda. Dried smears of epididymal spermatozoa were stained with 1 % Eosin Y for 30 minutes and evaluated at 400 x magnification. Five hundred spermatozoa per rat were evaluated and scored as morphologically normal or abnormal according to the presence or absence of head or tail defects.
Histopathology	After fixation, samples were trimmed, processed, embedded in paraffin wax, sectioned to a thickness of 4-5 μm and then processed in an alcohol-xylene series and stained with hematoxylin and eosin for microscopic evaluation. Histopathology evaluation was performed blind by at least two pathologists. At least one senior pathologist peer reviewed all lesions of oncological interest as well as any lesion of dubious interpretation. All the pathologists used the same evaluation criteria and the same classification based on international standard criteria (INHAND, NTP) described in the specific Standard Operating Procedures and long adopted at the Cesare Maltoni Cancer Research Centre/Ramazzini Institute (CMCRC/RI).
Hormone analysis	Serum concentration of free (fT) and total testosterone (TT), 5α-dihydrotestosterone (DHT), 17β-estradiol (E2) and Sex Hormone Binding Globulin (SHBG) were measured in duplicates by solid phase enzyme-linked immunosorbent assays (ELISAs): "Estradiol rat ELISA" (#DEV9999), manufactured by Demeditec Diagnostics GmbH, "Rat Free Testosterone (F-TESTO) ELISA" (#CSB-E0597r), "Rat Testosterone, T ELISA" (#CSB-E05100R); "Rat dihydrotestosterone (DHT) ELISA" (#CSB-E07879r), and "Rat sex hormone-binding globulin (SHBG) ELISA" (#CSB-E12118r), manufactured by Cusabio Biotech Co. Ltd. The detection range and the Lower Limit of Detection (LLD) of each ELISA kit was 2.5-1280 pg/mL and 2.5 pg/mL for E2, 0.3-60 pg/mL and 0.15 pg/mL for fT, 0.13-25.6 ng/mL and 0.06 ng/mL for TT, 10-2000 pg/mL and 5 pg/mL for DHT, 375-6000 ng/mL and 375 ng/mL for SHBG. Each kit was used following the manufacturer's instructions and absorbance was measured at 450 nm using a 96-well plate reader. Plasma pituitary hormones were measured in duplicate using the "Rat Pituitary Magnetic Bead Panel", a Luminex® bead-based immunoassay, following manufacturers' instructions. Seven plasma pituitary hormones were measured in plasma samples from 40 pups (20 females and 20 males) randomly selected from the 6–week cohort (N = 48 total): adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF), follicle stimulating hormone (FSH), growth hormone (GH), luteinizing hormone (LH), prolactin (PRL) and thyroid stimulating hormone (TSH). FSH and LH were also assessed in 40 pups (20 females and 20 males) randomly selected from the 13–week cohort (N = 60 total). BDNF and TSH results from the 6–week cohort showed marginal differences by exposure groups in male pups, it was therefore attempted to validate these results by measuring BDNF and TSH in all male pups (N = 30) from the 13–week cohort. Plasma TT was measured in duplicates in all dams (N = 24) using an ELISA kit, the "Testosterone Parameter Assay Kit", following manufacturers' instructions.
Statistical methods	Where data on a particular endpoint were collected from both sexes, analyses were conducted separately. All statistical tests were made using a significance level of $\alpha = 0.05$. For continuous data including body weight, weight gain and organ weights, which are most often normally distributed, one-way ANOVA, followed by a Dunnett's test was used to

	compare treatment versus control groups. For hormone data, which are usually non-normally distributed and have high inter-individual variability, a screening for outliers was made, based on a Box and Whisker Plot procedure and considering as outliers the values that were outside the box boundaries by more than 3 times the size of the box itself. In the case of hormone ratios, the same outliers of the single evaluation were considered. Nonparametric Kruskal-Wallis' tests, using beta approximation, were used in cases where data were not normally distributed (all hormones). Counting data, not normally distributed, were also analysed with appropriate regression models. Where the observations were grouped (such as for litter data), fixed and mixed effect models were estimated (litter as random effect) and both reported. For biological parameters related to the body weight (such as the AGD), statistical analyses were always performed including the body weight of each pup in the regression model. The incidence of pathological lesions, reported as the numbers of animals bearing lesions, were compared using a two-tail Fisher exact test. The statistical analysis was performed using Stata/IC 10.1 (for all regressions) and Statisti× 10 (for all the other tests); graphs were obtained using Microsoft Excel and Statisti× 10.

Results

No statistically significant differences were observed between the control and the glyphosate and Roundup Bioflow (MON 52276) groups for gestational index, mean gestational length, relative weight gain during pregnancy, relative weight gain during lactation, total number of pups delivered at PND 0, litter size, sex ratio, mean life birth index, number of dams with reported stillbirths (although higher in glyphosate group (4/8) compared to control (2/8)), number of still born pups, and survival index on PND 1 and PND 21 (Table 1). Also no treatment related effects were observed for water or feed consumption during gestation or lactation. In pups, AGD on PND 4 was statistically significantly increased both in MON 52276 treated males and females and in glyphosate treated males. Results were still significant after running multilevel linear regression models adjusted for body weight and litter as a random effect. Post-weaning body weights as well as water and feed consumption showed no difference in both female and male offspring. In female offspring, age and body weight at vaginal opening (VO) was similar across treatment groups, however, age at first estrous (FE) was statistically significantly delayed in the MON 52276 exposed group (Table 2). Female offspring in the control and glyphosate treated groups presented the FE within 6 days from the VO, while in the MON 52276 treated group 2/10 females presented a more than doubled interval (12 and 14 days) between VO and FE. In female pups followed up to 13–weeks (N = 30), the percent of time spent in each stage of the estrous cycle did not differ between glyphosate and MON 52276 treated animals and controls (Table 3). In male offspring, exposure to glyphosate or MON 52276 did not affect the time to balano-preputial separation (BPS) or sperm parameters (number of mature spermatids in the testis, daily sperm production, number and sperm transit time through caput/corpus and cauda epididymis and morphology) (Tables 2 and 4). There were no treatment-related gross lesions in F0 and F1 reproductive and endocrine organs in either sex and there was no statistically significant effect on absolute and relative organ weight of adrenal glands, uterus and ovaries in the dams, adrenal glands, testis, epididymis, bladder/prostate and seminal vesicles/coagulating glands in male offspring (with the exception of a decrease in absolute epididymal weight in the 13–week cohort), and adrenal glands, uterus and ovaries in female offspring (Tables 5, 6 and 7).

Most pituitary hormones were unaffected by exposure to glyphosate or MON 52276 in males, with the exception of a statistically significant increase in plasma TSH in the glyphosate group of the 6–week cohort and the MON 52276 group of the 13–week cohort and a statistically significant increase in plasma brain-derived neurotrophic factor (BDNF) in the MON 52276 group of the 6–week cohort. Apart from a statistically significant decrease in serum DHT in MON 52276 treated males of the 13–week cohort and a statistically significant increase in serum total testosterone in MON 52276 treated females of the 13–week cohort no effects were found on sex hormones (Tables 8 and 9). Hormone ratios were calculated as indicators of the general balance between hormones and sex steroid hormone bioavailability. The TT/SHBG ratio was statistically significantly increased in MON 52276 treated females of the 13–week cohort. The E2/SHBG ratio was statistically significantly increased in MON 52276 treated males of the 6–week cohort. The fT/TT ratio was statistically significantly decreased in glyphosate treated males of the 6–week cohort and in MON 52276 treated males of the 13–week cohort. Male and female MON 52276 treated rats of the 13–week cohort showed a marked and statistically significant decrease in DHT/TT ratio. No such differences were observed for DHT/TT ratio in males and females of the 6–week cohort and no statistically significant differences were observed for the E2/TT ratio in males and females of the glyphosate and MON 52276 treated groups of both cohorts (Tables 10 and 11).

Table 1 Maternal and reproductive outcome of dams exposed to glyphosate or Roundup Bioflow throughout pregnancy and lactation

Parameter	Control	Glyphosate	Roundup
Gestational index (%)[a]	100 (8/8)	100 (8/8)	100 (8/8)
Mean gestational length (day)[b]	22.9	23.0	23.0
Relative weight gain during pregnancy (%)[d, e]	33.1 ± 1.8	32.4 ± 2.2	33.2 ± 1.4
Relative weight gain during lactation (%)[d, f]	3.1 ± 0.7	2.5 ± 0.5	2.9 ± 0.8
Total pups (n) delivered at PND 0[g]	120	115	124
Litter size (n)[d, h]	15 ± 1.3	14.4 ± 1.9	15.5 ± 1.7
Sex ratio at birth (%)[d, i]	53.6 ± 16.9	43.2 ± 9.9	45.4 ± 12.6
Mean live birth index (%)[d, l]	95.9 ± 9.4	93.9 ± 6.8	96.1 ± 5.8
Dams with reported stillbirths (n)	2	4	3
Stillborn (n)[m]	5	7	5
Survival index at PND 1 (%)[n]	90.8 ± 10.6	93.0 ± 8.3	91.5 ± 9.0
Survival index at PND 21 (%)[n]	90.0 ± 10.0	91.3 ± 8.3	88.1 ± 8.0

[a]Gestational index = (number of females with live born / number of females with evidence of pregnancy) × 100
[b]Mean gestational length = mean number of days between GD 0 (day of positive evidence of mating) and day of parturition
[d]Mean ± standard deviation
[e]Relative weight gain during pregnancy = relative weight on the last day of pregnancy minus relative weight on the first day treatment in pregnancy, i.e. GD 6 (weight on GD 6 = 100%)
[f]Relative weight gain during lactation = relative weight on LD 21 minus relative weight on the first day of lactation, i.e. LD 1 (weight on LD 1 = 100%)
[g]Live and stillborn pups are considered
[h]Mean number of pups per litter at PND 0 (within 24 h from delivery)
[i]Sex ratio at birth = (no. of male offspring / no. of total offspring) × 100
[l]Live birth index = (no. of offspring born alive / no. of offspring born) × 100
[m]Stillborn = no. of pups died in utero
[n]Survival index = (no. of live offspring at designated time-point / no. of pups born) × 100

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 2 Effects of glyphosate or Roundup Bioflow exposure on developmental landmarks and sexual characteristics of pups

Parameter	Control	Glyphosate	Roundup
Number of male pups at PND 1	58	46	53
Male pups weight at PND 1 (g)[a]	6.8 ± 0.5	7.1 ± 0.2	6.8 ± 0.4
Male pups weaning weight (g)[a, b]	50.4 ± 4.4	53.5 ± 6.0	51.8 ± 5.8
Male AGD (mm) at PND 4[a, c]	4.02 ± 0.49	4.26 ± 0.38**	4.34 ± 0.30**°°
Age (PND) at balano-preputial separation (BPS)	46.33 ± 1.85	46.78 ± 1.73	47.61 ± 2.77
Body weight at BPS (g)	202.50 ± 10.74	203.89 ± 16.68	207.50 ± 22.70
Number of female pups at PND 1	51	61	60
Female pups birth weight (g)[a]	6.4 ± 0.4	6.6 ± 0.4	6.5 ± 0.6
Female pups weaning weight (g)[a, b]	48.3 ± 5.1	50.4 ± 5.2	50.5 ± 5.1
Female AGD (mm) at PND 4[a, c]	1.70 ± 0.25	1.79 ± 0.21	1.86 ± 0.19**°°
Age (PND) at vaginal opening (VO)[a]	35.56 ± 1.72	35.39 ± 1.5	35.61 ± 1.14
Body weight at VO (g)[a]	108.33 ± 6.18	108.06 ± 7.10	109.44 ± 8.73
Age (PND) at First Estrous (FE)[a, d]	39.88 ± 1.25	40.13 ± 1.46	42.63 ± 3.25#
Number of days between VO and FE[a]	4.75 ± 0.71	5.13 ± 0.64	7.00 ± 3.78

**Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight
°°Statistically significant ($p < 0.01$) with multilevel linear regression adjusted for body weight and litter (random effect)
#Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
[a]Mean ± standard deviation
[b]Weaning weight corresponds to PND 25
[c]AGD = ano-genital distance
[d]First estrous (FE) was evaluated only in females belonging to the 6 week cohort

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 3 Estrous cycle characterization in female rats belonging to the 13-week cohort

Time (%) in cycle stages	N. females	Control	Glyphosate	Roundup
Time (%) in Diestrus	10	55.24 ± 11.70	51.43 ± 5.41	52.86 ± 5.24
Time (%) in Proestrus	10	20.95 ± 7.51	23.33 ± 5.24	23.86 ± 3.76
Time (%) in Estrus	10	23.33 ± 5.24	25.24 ± 3.92	23.81 ± 2.24

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 4 Effects of glyphosate or Roundup Bioflow exposure on sperm parameters

Parameter	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Sperm number ($\times 10^6$/g testis)[a]	90.3 ± 22.0	83.7 ± 15.6	81.0 ± 12.0	109.4 ± 17.9	107.9 ± 11.3	119.6 ± 20.2
Daily sperm production ($\times 10^6$/g testis)[a]	14.8 ± 3.6	13.7 ± 2.6	13.3 ± 2.0	17.9 ± 2.9	17.7 ± 1.8	19.6 ± 3.3
Cauda epididimal sperm number ($\times 10^6$)[a]	51.2 ± 10.5	51.9 ± 10.5	50.9 ± 10.7	129.5 ± 28.5	125.9 ± 14.0	122.7 ± 11.0
Caput/corpus epididimal sperm number ($\times 10^6$)[a]	67.6 ± 6.1	66.5 ± 7.9	66.5 ± 10.6	97.0 ± 16.4	93.3 ± 13.0	93.2 ± 8.8
Sperm transit time through caput + corpus of epididymis (days)[a]	4.8 ± 1.3	5.0 ± 1.1	5.1 ± 1.0	5.5 ± 0.8	5.3 ± 1.0	4.8 ± 0.7
Sperm transit time through cauda of epididymis (days)[a]	3.5 ± 0.5	3.8 ± 0.8	3.9 ± 0.8	7.4 ± 1.8	7.2 ± 0.9	6.4 ± 1.1
Sperm transit time through epididymis in toto (days)[a]	8.3 ± 1.7	8.8 ± 1.5	8.9 ± 1.8	12.8 ± 2.2	12.5 ± 1.7	11.2 ± 1.7
Total abnormal sperm (%)[a]	6.3 ± 1.3	6.4 ± 1.8	6.3 ± 1.4	4.6 ± 1.4	4.3 ± 1.9	3.5 ± 1.3

[a]Mean ± standard deviation

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 5 Organ weights and testosterone level in dams

	Control	Glyphosate	Roundup
No. of dams examined	8	8	8
Body weight (g)[a]	302 ± 10	306 ± 15	317 ± 13
Adrenal glands[a, b]	0.110 ± 0.030 [0.036 ± 0.009]	0.106 ± 0.013 [0.035 ± 0.005]	0.106 ± 0.045 [0.033 ± 0.005]
Uterus[a, b]	0.867 ± 0.285 [0.286 ± 0.091]	0.772 ± 0.129 [0.253 ± 0.046]	0.994 ± 0.239 [0.298 ± 0.076]
Ovaries[a, b]	0.239 ± 0.62 [0.079 ± 0.018]	0.235 ± 0.047 0.077 ± 0.017]	0.247 ± 0.052 [0.078 ± 0.016]
TT (ng/ml)[a]	3.77 ± 0.53	4.24 ± 1.48	3.90 ± 0.56

[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 6 Organ weights of male offspring

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8	8	8	10	10	10
Body weight (g)[a]	321 ± 19	326 ± 16	317 ± 23	458 ± 19	454 ± 19	449 ± 15
Adrenal glands[a, b]	0.070 ± 0.014 [0.022 ± 0.005]	0.071 ± 0.015 [0.022 ± 0.004]	0.063 ± 0.009 [0.020 ± 0.002]	0.085 ± 0.025 [0.019 ± 0.005]	0.083 ± 0.024 [0.018 ± 0.005]	0.144 ± 0.190 [0.032 ± 0.041]
Testis[a, b]	1.475 ± 0.052 [0.461 ± 0.023]	1.474 ± 0.077 [0.453 ± 0.023]	1.459 ± 0.100 [0.460 ± 0.017]	1.568 ± 0.065 [0.342 ± 0.013]	1.529 ± 0.076 [0.337 ± 0.013]	1.562 ± 0.068 [0.347 ± 0.012]
Epididymis[a, b]	0.370 ± 0.041 [0.115 ± 0.011]	0.343 ± 0.040 [0.105 ± 0.010]	0.368 ± 0.038 [0.116 ± 0.006]	0.595 ± 0.039 [0.130 ± 0.008]	0.563 ± 0.043 [0.124 ± 0.008]	0.553 ± 0.026* [0.123 ± 0.006]
Bladder/Prostate[a, b]	0.563 ± 0.080 [0.176 ± 0.026]	0.561 ± 0.099 [0.173 ± 0.036]	0.506 ± 0.090 [0.159 ± 0.022]	0.967 ± 0.086 [0.211 ± 0.024]	0.897 ± 0.160 [0.197 ± 0.033]	0.906 ± 0.190 [0.202 ± 0.045]
Seminal vesicles and coagulating gland[a, b]	1.129 ± 0.129 [0.353 ± 0.045]	1.110 ± 0.291 [0.339 ± 0.083]	1.235 ± 0.135 [0.389 ± 0.029]	2.049 ± 0.418 [0.446 ± 0.083]	1.879 ± 0.298 [0.414 ± 0.063]	2.055 ± 0.404 [0.457 ± 0.090]

*Statistically significant with Dunnett's test ($p < 0.05$)
[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio *100)

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 7 Organ weights (g) of female offspring

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8	8	8	10	10	10
Body weight (g)[a]	225 ± 13	219 ± 11	223 ± 11	280 ± 20	283 ± 13	283 ± 13
Adrenal glands[a, b]	0.081 ± 0.012 [0.036 ± 0.005]	0.073 ± 0.009 [0.033 ± 0.004]	0.079 ± 0.012 [0.036 ± 0.005]	0.094 ± 0.019 [0.033 ± 0.005]	0.084 ± 0.019 [0.030 ± 0.007]	0.086 ± 0.018 [0.031 ± 0.006]
Uterus[a, b]	0.499 ± 0.114 [0.221 ± 0.059]	0.531 ± 0.162 [0.241 ± 0.069]	0.619 ± 0.221 [0.277 ± 0.099]	0.539 ± 0.082 [0.192 ± 0.025]	0.575 ± 0.137 [0.202 ± 0.047]	0.589 ± 0.111 [0.209 ± 0.043]
Ovaries[a, b]	0.181 ± 0.024 [0.081 ± 0.012]	0.169 ± 0.027 [0.077 ± 0.013]	0.172 ± 0.034 [0.077 ± 0.014]	0.192 ± 0.029 [0.068 ± 0.009]	0.182 ± 0.025 [0.064 ± 0.009]	0.186 ± 0.036 [0.065 ± 0.012]

[a]Mean ± standard deviation
[b]Absolute organ weight (g). In square brackets relative organ weight (organ weight / body weight ratio × 100)

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 8 Effects of glyphosate or Roundup Bioflow exposure on hormones in males (mean ± SEM)

	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
Serum Hormones						
No. of males examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
TT (ng/ml)	1.12 ± 0.12	1.02 ± 0.28	0.84 ± 0.11[a]	8.16 ± 2.86	7.65 ± 2.86	3.76 ± 0.90
fT (pg/ml)	14.53 ± 2.37	7.45 ± 2.23[b]	13.12 ± 3.74[b]	296.70 ± 123.70[c]	724.24 ± 419.22[c]	90.40 ± 29.44
DHT (pg/ml)	761.11 ± 136.21	575.28 ± 238.24	554.29 ± 145.16[a]	15,709.0 ± 5547.20	16,711.8 ± 6724.5	1980.2 ± 664.68[c**]
SHBG (ng/ml)	861.20 ± 30.24	833.24 ± 21.15	856.78 ± 32.39	917.58 ± 16.94	906.36 ± 21.62	906.51 ± 18.89
E2 (pg/ml)	1.04 ± 0.21[a]	3.29 ± 1.85	6.19 ± 2.28[b]	3.66 ± 2.57[c]	1.08 ± 0.02[d]	6.00 ± 1.11
Plasma Hormones						
No. of males examined	7 (8)	6 (8)	7 (8)	10 (10)	10 (10)	10 (10)
FSH (ng/ml)	7.00 ± 1.38	6.43 ± 1.16	7.18 ± 0.68	2.32 ± 0.40[e]	2.18 ± 0.16[f]	2.90 ± 0.28[f]
LH (ng/ml)	3.76 ± 0.79	2.87 ± 0.63	4.41 ± 0.62	1.20 ± 0.17[e]	1.25 ± 0.24[d]	1.40 ± 0.18[f]
PRL (ng/ml)	3.83 ± 0.64	3.00 ± 0.64	4.31 ± 1.32	–	–	–
GH (ng/ml)	6.03 ± 4.32[g]	23.19 ± 21.17[h]	4.38 ± 1.94[i]	–	–	–
TSH (ng/ml)	4.23 ± 0.76	8.17 ± 1.58*	5.57 ± 0.31[b]	1.89 ± 0.20	2.53 ± 0.25	3.69 ± 0.42**
ACTH (pg/ml)	346.67 ± 35.52	255.18 ± 43.29	292.26 ± 26.22	–	–	–
BDNF (pg/ml)	99.49 ± 25.32[b]	148.85 ± 37.53	171.79 ± 14.65*	53.83 ± 14.77[c]	58.07 ± 13.83	45.15 ± 14.64

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]7 out 8
[b]6 out 8
[c]9 out 10
[d]8 out 10
[e]6 out 10
[f]7 out 10
[g]5 out 8
[h]3 out 8
[i]4 out 8

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 9 Effects of glyphosate or Roundup Bioflow exposure on hormones in female (mean ± SEM)

	6 week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
Serum Hormones						
No. of females examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
TT (ng/ml)	0.66 ± 0.064	0.75 ± 0.12	0.68 ± 0.11[a]	0.51 ± 0.06	0.72 ± 0.10	0.72 ± 0.07[b] *
fT (pg/ml)	6.49 ± 1.00[c]	6.74 ± 1.89[d]	7.70 ± 1.35[a]	9.18 ± 2.49	12.04 ± 1.25	12.52 ± 1.76[b]
DHT (pg/ml)	294.28 ± 50.40	328.34 ± 51.93[a]	488.94 ± 114.68[a]	382.93 ± 52.14	460.09 ± 60.06	268.84 ± 45.56[b]
SHBG (ng/ml)	864.82 ± 30.24	952.75 ± 54.98	903.07 ± 29.61	968.27 ± 21.39	993.44 ± 32.79	964.81 ± 27.20
E2 (pg/ml)[g]	14.95 ± 7.24	32.24 ± 8.77	66.96 ± 25.17	18.08 ± 8.49	28.48 ± 13.71	43.91 ± 9.92
Plasma Hormones						
No. of females examined	7 (8)	7 (8)	6 (8)	7(10)	5(10)	6(10)
FSH (ng/ml) [g]	3.95 ± 2.50	2.67 ± 1.22	3.15 ± 1.65	1.58 ± 0.51	1.73 ± 0.64	1.46 ± 0.35
LH (ng/ml)[g]	5.75 ± 3.04	4.86 ± 1.93	4.52 ± 3.38	1.83 ± 0.25	2.35 ± 1.11	2.16 ± 1.28
PRL (ng/ml)[g]	102.34 ± 164.71[c]	27.49 ± 30.23	46.49 ± 31.03	–	–	–
GH (ng/ml)	12.61 ± 13.30	3.85 ± 0.97[c]	4.16 ± 2.84	–	–	–
TSH (ng/ml)	2.70 ± 1.13	3.02 ± 2.00	3.04 ± 1.53	1.29 ± 0.69[e]	1.93 ± 0.89[f]	3.03 ± 2.22[e]
ACTH (pg/ml)	331.60 ± 89.59	314.09 ± 170.60	354.95 ± 104.96	–	–	–
BDNF (pg/ml)	245.03 ± 155.68	483.62 ± 301.02	351.33 ± 177.28	25,399 ± 155.77[e]	377.79 ± 226.30[f]	249.39 ± 14,566[e]

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]7 out 8
[b]9 out 10
[c]6 out 8
[d]5 out 8
[e]4 out 10
[f]2 out 10
[g]Not statistically evaluated due to insufficient sample size after clustering on the basis of the estrous cycle

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 10 Effects of glyphosate or Roundup Bioflow exposure on hormone ratios in males (Mean ± SEM)

Hormone ratios (ng/ml)	6-week cohort			13-week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of males examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	10 (10)
fT/TT (x 10^{-3})	12.5 ± 1.35	8.67 ± 0.83[a] *	10.8 ± 0.45[b]	53.0 ± 11.1[c]	85.0 ± 27.5[c]	24.7 ± 4.03*
DHT/TT	0.658 ± 0.081	0.511 ± 0.087	0.614 ± 0.122[d]	2.195 ± 0.37	2.490 ± 0.70	0.65 ± 0.15[c] **
E2/TT (x 10^{-3})	1.01 ± 0.19[d]	2.65 ± 1.15	10.1 ± 5.17[d]	1.59 ± 1.23[c]	0.52 ± 0.14[e]	1.84 ± 0.42[c]
TT/SHBG (x 10^{-4})	12.90 ± 1.35	12.23 ± 3.25	10.1 ± 1.37[d]	88.30 ± 31.86	86.59 ± 33.21	40.68 ± 9.36
E2/SHBG (x 10^{-6})	1.24 ± 0.23[d]	3.90 ± 2.21	7.18 ± 2.66*	3.87 ± 2.69[c]	1.20 ± 0.003[f]	6.66 ± 1.25

*Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
**Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]6 out 8
[b]5 out 8
[c]9 out 10
[d]7 out 8
[e]8 out 10
[f]7 out 10

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Table 11 Effects of glyphosate or Roundup Bioflow exposure on hormone ratios in females

Hormone ratios (ng/ml)	6 week cohort			13 week cohort		
	Control	Glyphosate	Roundup	Control	Glyphosate	Roundup
No. of females examined	8 (8)	8 (8)	8 (8)	10 (10)	10 (10)	9 (10)
fT/ TT (x 10^{-3})	9.17 ± 0.48[a]	8.12 ± 0.55[b]	11.90 ± 2.37[c]	19.3 ± 5.20	19.0 ± 2.80	17.0 ± 1.66
DHT/ TT	0.428 ± 0.036	0.481 ± 0.060[c]	0.718 ± 0.173[c]	0.794 ± 0.11	0.702 ± 0.10	0.383 ± 0.07[d**]
E2/ TT[e]	0.012 ± 0.0003	0.045 ± 0.014	0.066 ± 0.028[c]	0.019 ± 0.005	0.035 ± 0.012	0.068 ± 0.015[d]
TT/SHBG (x 10^{-4})	7.85 ± 1.02	8.00 ± 1.26	7.81 ± 1.35[c]	5.35 ± 0.62	7.27 ± 0.98	7.5 ± 0.61[*]
E2/SHBG (x 10^{-5})[e]	1.69 ± 0.78	3.51 ± 1.00	7.28 ± 2.55	1.90 ± 0.89	2.89 ± 1.41	4.87 ± 1.10 [d]

[*]Statistically significant ($p < 0.05$) with Kruskal-Wallis' tests
[**]Statistically significant ($p < 0.01$) with Kruskal-Wallis' tests
[a]6 out 8
[b]5 out 8
[c]7 out of 8
[d]9 out 10
[e]Not statistically evaluated due to insufficient sample size after clustering on the basis of the estrous cycle

(Table adapted from Manservisi *et al.* (2019). The Ramazzini Institute 13-week pilot study glyphosate-based herbicides administered at human-equivalent dose to Sprague Dawley rats: effects on development and endocrine system, Environmental Health (2019) 18:15 doi.org/10.1186/s12940-019-0453-y)

Conclusion

The present study demonstrates that exposure to MON 52276 at a dose level equivalent to 1.75 mg glyphosate acid/kg bw/day, from the prenatal period to adulthood, induced endocrine effects and altered reproductive developmental parameters in male and female SD rats. MON 52276 exposure was associated with androgen-like effects, in particular in females, including a statistically significant increase of ano-genital distance in both males and females, a delay of first estrous and increased testosterone in females. MON 52276 exposure was also associated with altered testosterone metabolism in both males and females, where a statistically significant decrease in DHT/TT ratio was observed in the longest treated group (13–week cohort). Overall, MON 52276 elicited more pronounced effects than glyphosate, which only increased anogenital distance and TSH concentration in male rats in the peripubertal window (6–week cohort).

Assessment and conclusion

Assessment and conclusion by applicant:

In this pilot study the effect of glyphosate and its reference formulation Roundup Bioflow (MON 52276) at a dose of 1.75 mg glyphosate acid eq./kg bw/day on endocrine modulation was investigated in female rats during pregnancy and lactation, and in male and female rats during lactation, the peripubertal period and adulthood. The endpoints analysed were body weight, water and food consumption, gestational parameters, litter parameters, landmarks of sexual development, estrous cyclicity, gross and histopathology of reproductive and endocrine tissues, sperm parameters and serum and plasma hormone levels. MON 52276 exposure was associated with statistically significant increase of ano-genital distance in males and females, a delay of first estrous and increased serum testosterone in females and altered testosterone metabolism in both males and females. MON 52276 elicited more pronounced effects than glyphosate, which only increased statistically significantly anogenital distance during the peripubertal period. The statistically significant increase in TSH levels in glyphosate and MON 52276 treated rats was not associated with histopathological changes in the thyroid and thus of minor toxicological significance. The effect of glyphosate on ano-genital distance is not corroborated by any reproductive toxicity study where rats were exposed to much higher doses of glyphosate (> 1,000 mg/kg bw/day).

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level tested.

Reliability criteria made by the applicant

Publication: Manservisi *et al.*, 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing	N	Pilot study based on OECD guideline 443 but with deviations.

guidelines		
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of >99.5 % as Pestanal™. Source: Sigma-Aldrich, Milan, Italy.
Only glyphosate acid or one of its salts is the tested substance	N	Also, representative formulated product tested. Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5 %), water (42.5 %) and surfactant (16 %)). Source: Consorzio Agrario dell'Emilia, Bologna, Italy.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral via the drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./bw/day.
Number of animals used per dose level reported	Y	Dams: 8/group. Offspring: 8 M + 8F/group (6–week cohort); 10 M + 10F/group (13–week cohort).
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Not possible, only comparison between glyphosate and MON 52276.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered as relevant for the risk assessment of glyphosate but reliable with restrictions because of the limited number of animals used per dose level and only one dose level was tested.		

Assessment and conclusion by RMS:

In this (pilot) study, two groups of Sprague-Dawley rats (8/group) were treated from gestation day 6 with either glyphosate (G) (> 99.5% pure) or Roundup Bioflow (R) (MON 52276) (360 g/L of glyphosate acid) diluted in drinking water to achieve glyphosate dose of 1.75 mg/kg bw/day (the US Acceptable Daily Intake). On post-natal day (PND) 28, the offspring were weaned and randomly distributed into two cohorts: 6-week cohort with 8 pups/sex/group and 13-week cohort with 10 pups/sex/group. After weaning, the pups also received the same dose of glyphosate or Roundup Bioflow as that of dams until their sacrifice (PND 73 ± 2 for the 6-week cohort;

and PND 125 ± 2 for the 13-week cohort). Reproductive outcome of dams, and developmental landmarks and sexual characteristics of pups were examined.

There were no statistically significant effects in dams on the following in both the G and R groups: body weight, water or feed consumption during gestation or lactation, gestational index, mean gestational length, total pups delivered at PND 0, litter size, sex ratio at birth, mean live birth index, dams with reported stillbirths (although the number of dams with stillbirths was higher in the glyphosate group (4/8) compared to control (2/8)), number of stillborns, survival index at PND 1 or 21, weight of adrenals, uterus or ovaries and total testosterone levels.

There were no statistically significant effects in pups on the following in both the G and R groups: weight at birth (females), at PND 1 (males) or at weaning; age or body weight at balano-preputial separation or vaginal opening; number of days between vaginal opening and first estrous; the time spent in each stage (diestrus, proestrus and estrus) of the estrous cycle; anogenital distance (AGD) in females at PND 4 (only in G group); and age at first estrous (only in G group). There were no statistically significant effects in male pups in both G and R groups in both 6-week and 13-week cohorts on body weight; organ weights (adrenals, testis, epididymis (except in R group 13-week cohort), bladder/prostate and seminal vesicles and coagulating gland); or on the sperm parameters examined (sperm number, daily sperm production, cauda or caput/corpus epididimal sperm numbers, sperm transit time through caput + corpus, cauda or epididymis in toto, and total abnormal sperm). There were no statistically significant effects in female pups in both G and R groups in both 6-week and 13-week cohorts on body weight and organ weights (adrenals, uterus and ovaries).

The AGD on PND 4 was statistically significantly increased in males of both G and R groups and also in females of R group. Also the age at first estrous was statistically significantly delayed in R group. The weight of epididymis in the 13-week cohort of the R group was statistically significantly decreased.

The following hormone analyses were made in pups in the 6-week and 13-week cohorts of both G and R groups: serum hormones – total testosterone (TT), free testosterone (fT), 5α-dihydrotestosterone (DHT), sex hormone binding globulin (SHBG), 17β-estradiol (E2); plasma hormones – follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), growth hormone (GH), thyroid stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), brain-derived neurotrophic factor (BDNF); and hormone ratios – fT/TT, DHT/TT, E2/TT, TT/SHBG, E2/SHBG.

There were statistically significant effects on the following hormones (ratios): increased TT in females of 13-week cohort of R group; increased DHT in males of 13-week cohort of R group; increased TSH in males of 6-week cohort of G group and in 13-week cohort of R group; increased BDNF in males of 6-week cohort of R group; decreased ft/TT in males of 6-week cohort of G group and of 13-week cohort of R group; decreased DHT/TT in males and females of 13-week cohort of R group; increased E2/SHBG in males of 6-week cohort of R group; and increased TT/SHBG in females of 13-week cohort of R group.
Overall, for glyphosate in this study, the effects were seen only in males pups and were limited to increased AGD, increased TSH (6-week cohort), and decreased fT/TT (6-week cohort). While T3 and T4 were not analysed in this study, the study authors state that in the absence of histological changes in the thyroid, the altered TSH is not indicative of thyroid-related activity. However, the RMS is of the view that the increased TSH should be considered as indicative of the thyroid-related activity at a low dose of glyphosate tested in this study and should be considered along with the outcome of thyroid parameters in other repeated dose toxicity studies with glyphosate. The increased AGD in male pups treated with glyphosate is indicative of androgenic activity. Such activity was more pronounced in the Roundup Bioflow treated groups indicated by increased AGD in both male and female pups, increased age at first estrous and increased total testosterone in female pups.

The study is relevant for risk assessment as it informs on endpoints relevant to reproductive and endocrine effects. The study is however reliable with restrictions because of the following reasons: only one (low) dose tested; small group sizes; blood sampling was done only once (at the end of life) and the timing (9 AM to 3 PM) of sampling could not rule out circadian-dependent modulation of circulating hormones.

Category A – Pham, T.H. *et al.*

Data point	CA 5.6.1/017
Report author	Pham, T.H. *et al.*
Report year	2019
Report title	Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice
Document No.	Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

The aim of the study was to investigate the possible impact of glyphosate and a glyphosate-based herbicide (Roundup 3 Plus) on the reproductive system of male mice.

Materials and methods

Test substance	Glyphosate (Sigma-Aldrich) and Glyphosate-based formulation Roundup 3 Plus (Monsanto Europe, Belgium) containing 229 g/l glyphosate isopropylamine salt (170 g/l glyphosate acid equivalent)
Lot/Batch	Not specified
Purity/Radiochemical purity	Glyphosate: ≥ 99.2%
Animals and dosing	Four-month-old outbred Swiss adult mice were exposed to glyphosate or glyphosate-based herbicide (Roundup 3 Plus) via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day from the day of vaginal plug detection (embryonic day 0.5) to 20 days post-partum (dpp). The control group received water alone. Control and treated mice of 5, 20, 35 days old and 8 months old were euthanised and the reproductive organs excised. At least 5 animals derived from at least 3 to 4 different litters in each group were used for this test.
Testosterone quantification	Serum was collected from ketamine/xylazine-anaesthetised adult animals by terminal cardiac exsanguination and aliquots were stored at -20 °C. Testosterone levels in the serum were assayed in duplicate using a commercial radioimmunoassay based on competitive binding with I125-labeled testosterone according to the manufacturer's recommendations.
Epididymal sperm count	The mice were euthanised and the epididymis excised, rapidly frozen in liquid nitrogen and stored at -80°C pending sperm count. The tissue was first cut in pieces and homogenised in 6 mL of 0.15 M NaCl containing 0.005 % (v/v) Triton X-100. After homogenisation by sonication, an aliquot of the cell suspension was loaded onto a Malassez hemocytometer, and spermatozoa heads were counted. The average sperm count was calculated from at least 6 controls or treated animals.
Histology and numbers of germ cells and Sertoli cells	Testis samples were fixed in Bouin's solution and embedded in paraffin. Histological sections (5 µm thick) were stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), only the adult animals were perfused and the testes were fixed for 24 hours in 4 % (w/v) paraformaldehyde and then embedded in paraffin. Testis sections (5 µm thick) were incubated overnight at 4°C with a primary rabbit polyclonal antibody DDX4 and then with secondary Alexa-Chicken anti-rabbit 488 antibody during 1 hour to check the number of undifferentiated spermatogonia in testes of mice of 5 days old. For IHC on testes of mice of 35 days old, testis sections (5 µm thick) were incubated overnight at 4 °C with goat anti-ZBTB16 and rat anti-GATA1 and then with secondary Alexa-chicken anti-goat 488 antibody and secondary Alexa-donkey anti-rat 594 during 1 hour. The sections were all counterstained with 0.001 % (v/v) 4,6-diamidino-2-phenylindole dihydrochloride and mounted in Vectashield before microscopic analysis. To quantify the number of Sertoli cells (GATA1-positive cells) and undifferentiated

	spermatogonia (ZBTB16-positive cells), the cells were manually counted in 30 sections on average of seminiferous epithelium at stage VII in controls and glyphosate treated groups. Cells in 3 different areas of the testis were analysed for each biological replicate.
RNA extraction and quantitative PCR	Total RNA was extracted from testes of animals of 5 days old using the RNeasy plus mini kit according the protocol of the manufacturer and reverse transcription was performed with 1 mg of RNA using the iScript cDNA Synthesis Kit according to the manufacturer's instructions. The resulting cDNA was diluted 5 times and used for quantitative PCR. QPCR was performed using the iTaq Universal SYBR Green Supermix according to the manufacturer's instructions on a CFX384 Touch Real-Time PCR Detection system. The PCR amplification of the coding regions of Actb and Rplp0 was used for normalisation. The data from at least 6 samples were analysed, compared, plotted, and expressed as a fold change in treated samples compared with controls.
Statistical analysis	Statistical tests were carried out using R software. For each experiment, the results were separated and compared by modality. First, the data were tested for normality by the Shapiro test and homoscedasticity by the Bartlett test. If the data distribution for each modality followed a normal distribution and if the variances were equal, the ANOVA test was performed, a parametric comparison test. If the data of at least 1 of the groups were not normally distributed or if the data of the groups were distributed normally but the variances were not equal, the Kruskal-Wallis test was carried out, a nonparametric comparison test of average. The differences were considered statistically significant when $p < 0.05$. In this case, the Tukey test was performed after the ANOVA test and the Mann-Whitney test after the Kruskal-Wallis test. In animals of 5 and 20 days old and 8 months old, data were collected by randomising in view of origin litter and pups were used as the experimental unit for statistical analysis. In animals of 35 days old, corresponding to the first complete wave of spermatogenesis, litters were used as the experimental unit for statistical analysis.

Results

Male reproductive parameters of prepubertal mice of 5 days old - No statistically significant change has been found in the number of spermatogonia of mice exposed to glyphosate at 0.5, 5 and 50 mg/kg bw/day. No statistically significant change has been found in RNA expression of Cyp11a1, Cyp19a1 and Ret at all dose levels tested. Statistically significant changes were observed in RNA expression of Bax (increase at 0.5 mg/kg bw only), Bcl2 (increase at all dose levels), Dazl (increase at 0.5 mg/kg bw/day only), Kit (decrease at 50 mg/kg bw/day only), Sall4 (decrease at all dose levels), Nanos3 (increase at 0.5 and 50 mg/kg bw/day but not at 5 mg/kg bw/day) and Foxo1 (increase at 0.5 mg/kg bw/day only) (Fig. 4).

Male reproductive parameters of mice of 20 days old - The histopathological analysis of testis sections revealed that glyphosate causes an adverse effect on testis morphology when compared with the control. An increase in vacuoles in the seminiferous epithelium was observed at all dose levels. Empty seminiferous tubules were seen at 5 mg/kg bw/day but not at 0.5 and 50 mg/kg bw/day glyphosate (Fig. 2).

Male reproductive parameters of mice of 35 days old - No statistically significant change has been found in relative epididymis weight, relative seminal vesicles weight, epididymal sperm count and GATA1 positive cells. A decrease in relative testes weight was recorded at 0.5 mg/kg bw/day glyphosate but not at the other dose levels. Serum testosterone was found to be statistically significantly decreased at 0.5 and 50 mg/kg bw/day but not at 5 mg/kg bw/day glyphosate (Fig. 1). The number of ZBTB16 positive cells were statistically significantly decreased at 5 mg/kg bw/day glyphosate but not at 0.5 and 50 mg/kg bw/day (Fig. 3).

Male reproductive parameters of mice of 8 months old - No statistically significant changes were noted for relative epididymis weight, relative seminal vesicles weight and serum testosterone. A statistically significant decrease has been observed for relative testes weight of mice exposed to glyphosate at 0.5 mg/kg bw/day but not at the higher dose levels (Fig. 5).

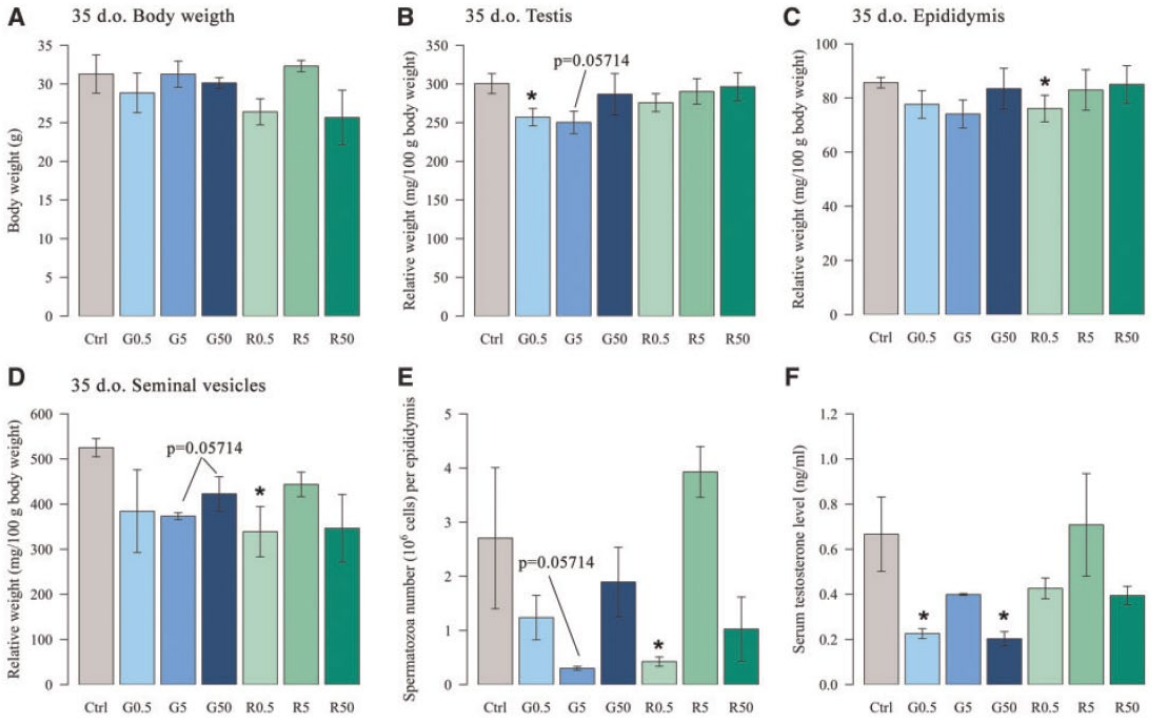

Figure 1. Perinatal exposure to glyphosate and/or GBH affects testis, epididymis and seminal vesicles weight, the number of spermatozoa and the secretion of testosterone in 35 d.o. mice. A, Body weight; B, testis; C, epididymis; D, seminal vesicles weight in 35 d.o. mice derived from females treated with vehicle (Ctrl; in gray), glyphosate (G0.5, G5, G50; in blue), and a GBH (R0.5, R5, R50; in green). E, Spermatozoa number in epididymis. F, The amount of testosterone levels in serum decreased in G0.5 and G50 groups compared with control. Values are expressed as mean value ± standard error. Ctrl, G0.5, G50, R0.5, R5: $n = 4$ litters (with 6 pups by litters); G5, R50: $n = 3$ litters (with 5 and 7 pups by litters, respectively). Statistical analyses were performed on the n number of litters using Kruskal-Wallis test followed by Mann-Whitney post hoc test to compare the control and treated groups, $^{*}p < .05$, is considered to be significantly different from the control group.

(Figure adapted from Pham *et al.* (2019). Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice. Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039)

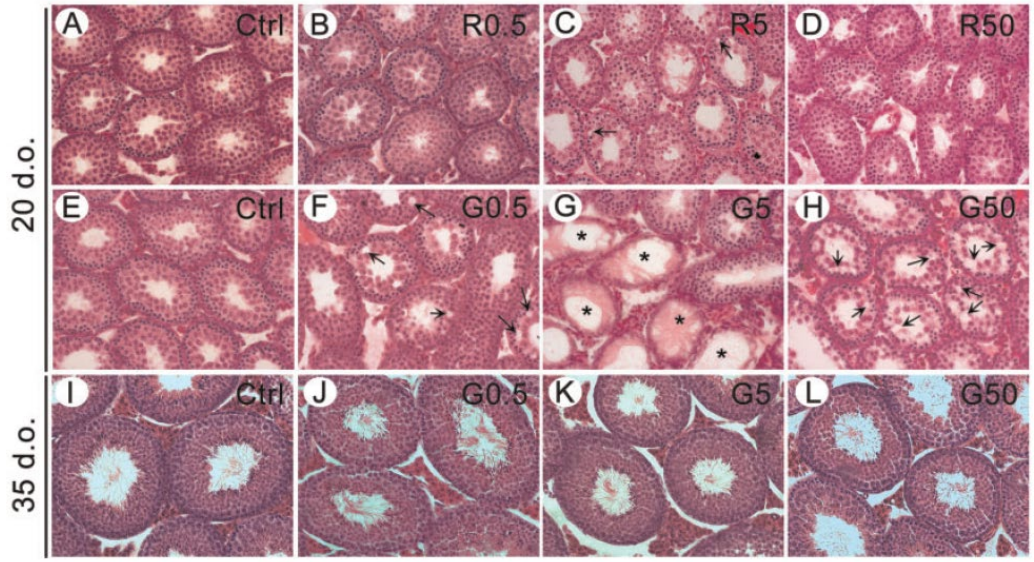

Figure 2. Perinatal exposure to glyphosate and not to a GBH affects the testis morphology in 20 d.o. mice. H&E staining of the histological sections in (A–H) 20 and (I–L) 35 d.o. mice from (A, E, and I) ctrl, (B) R0.5, (C) R5, (D) R50, (F and J) G0.5, (G and K) G5, and (H and L) G50, experimental groups. An increase in vacuoles in the seminiferous epithelium of the testis (arrow) and empty tubules (star) were observed in glyphosate group (G0.5, G5, and G50) compared with control. The presence of vacuoles were observed in R5 group as well. In 20 d.o. mice, the n number of progeny is $n = 5$ for each conditions, in 35 d.o. mice, Ctrl, R0.5, R5, R50, G0.5: $n = 6$; G5: $n = 5$; G50: $n = 7$.

(Figure adapted from Pham *et al.* (2019). Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice. Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039)

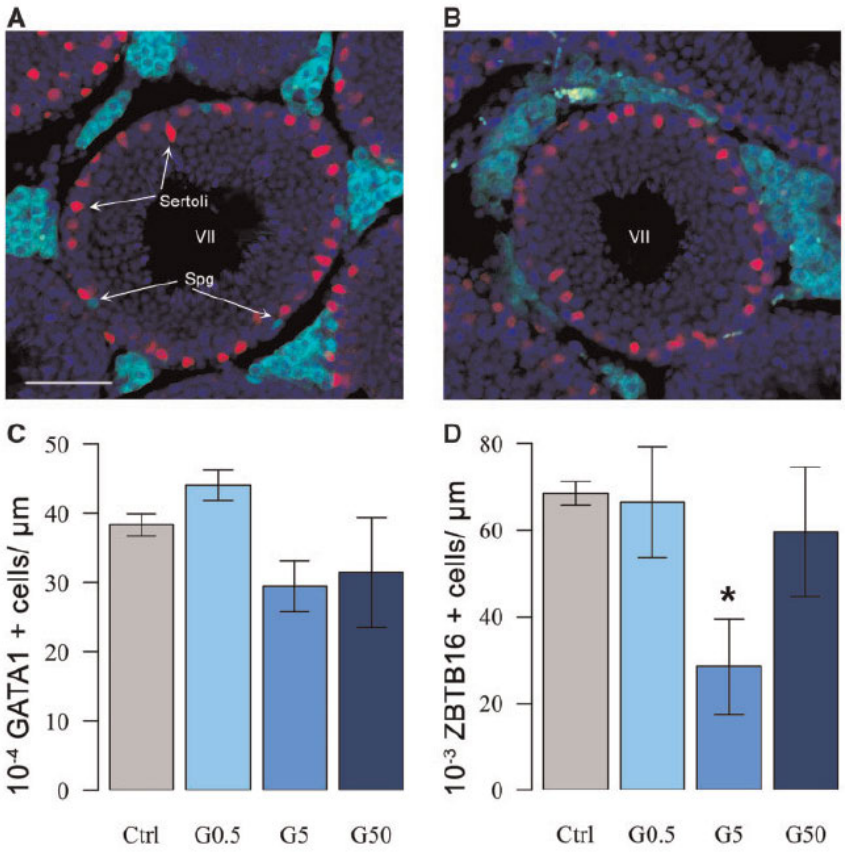

Figure 3. Perinatal exposure to glyphosate decreases the number of undifferentiated spermatogonia in adult mice. Representative images of testes sections from (A) the control (left panel) and (B) glyphosate (G5; right panel) animals: Sertoli cells and spermatogonia (Spg) were immunostained using anti-GATA1 (red) or anti-ZBTB16 (green) antibodies, respectively. The ZBTB16 antibody staining of Leydig cells located outside of seminiferous tubules is nonspecific. A quantitative analysis of (C) Sertoli cells and (D) spermatogonia was performed by manually counting the GATA1 and ZBTB16 positive cells at stage VII of the seminiferous epithelium. The contour of each tubule section was measured using ImageJ. The values shown indicate the cell counts per micrometers of tubule circumference. Values are mean ± standard error; $n = 5$ for all conditions. Statistical analyses were performed on the n number of progeny using Kruskal-Wallis test followed by Mann-Whitney post hoc test to compare the control and treated groups. $^{*}p < .05$ is considered to be significantly different compared with the control group. Scale bar 150 µm. The immunostaining of the testis sections was performed as described in the Materials and Methods section.

(Figure adapted from Pham *et al.* (2019). Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice. Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039)

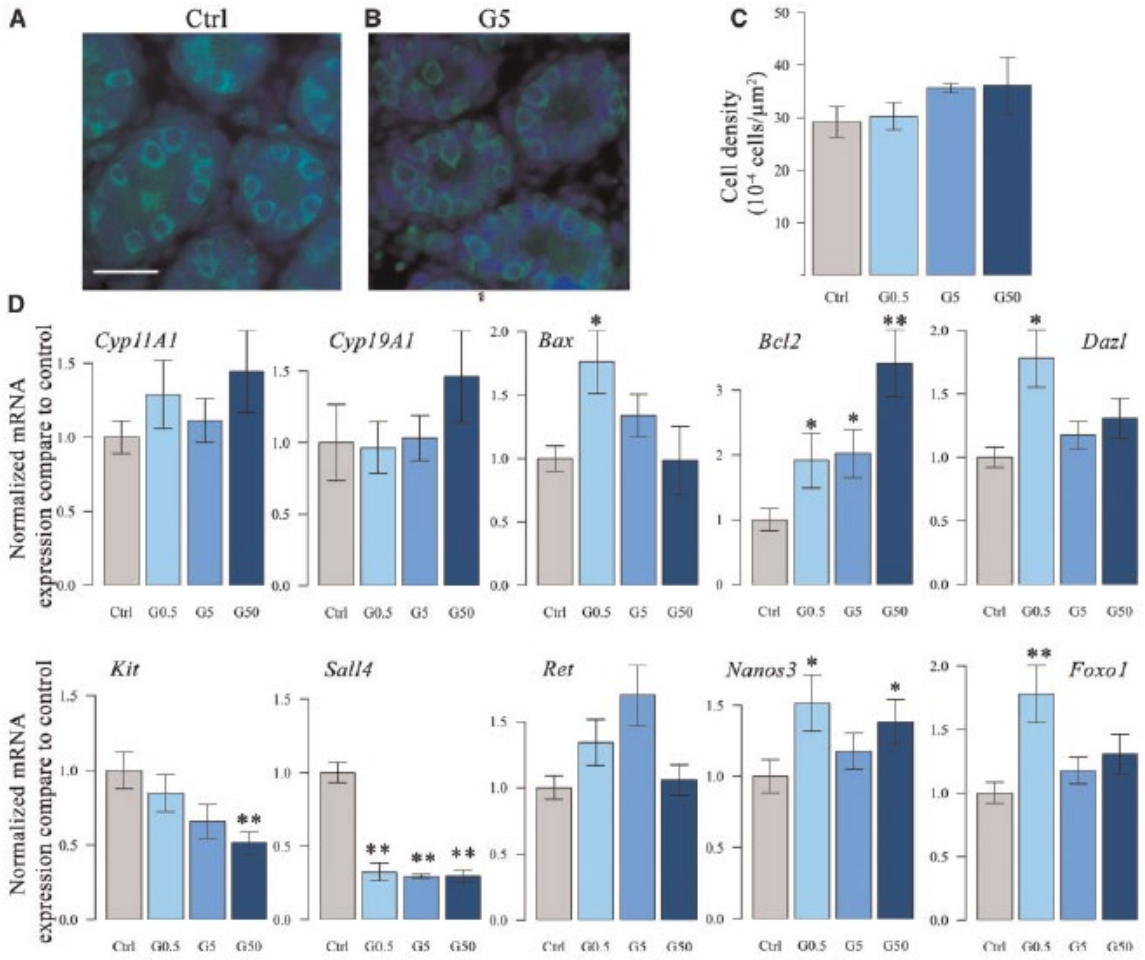

Figure 4. Perinatal exposure to glyphosate affects the expression of the genes involved in spermatogonia differentiation in prepubertal 5 d.o. mice. Representative images of testes sections from (A) the control (left panel) and (B) glyphosate (G5; right panel) animals: Spermatogonia were immunostained using anti-DDX4 (green) antibodies. Scale bar: 40 µm. C, Quantitative analysis of the number of spermatogonia showed no any significant differences in 5 d.o. testis. D, RT-QPCR analysis of RNA expression in control (Ctrl; in gray) and glyphosate groups (G0.5, G5, G50; in blue). Values are mean ± standard error. Ctrl, G5, G50: $n = 6$; G0.5: $n = 7$. Statistical analyses were performed on the n number of progeny using Kruskal-Wallis test followed by Mann-Whitney post hoc test to compare the control and a treated groups. $^{*}p < .05$, $^{**}p < .01$ are considered to be significantly different compared with control. The copy numbers of each target gene were normalized to *Actb* and *Rplp0*. Data were presented as normalized values compared with control.

(Figure adapted from Pham *et al.* (2019). Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice. Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039)

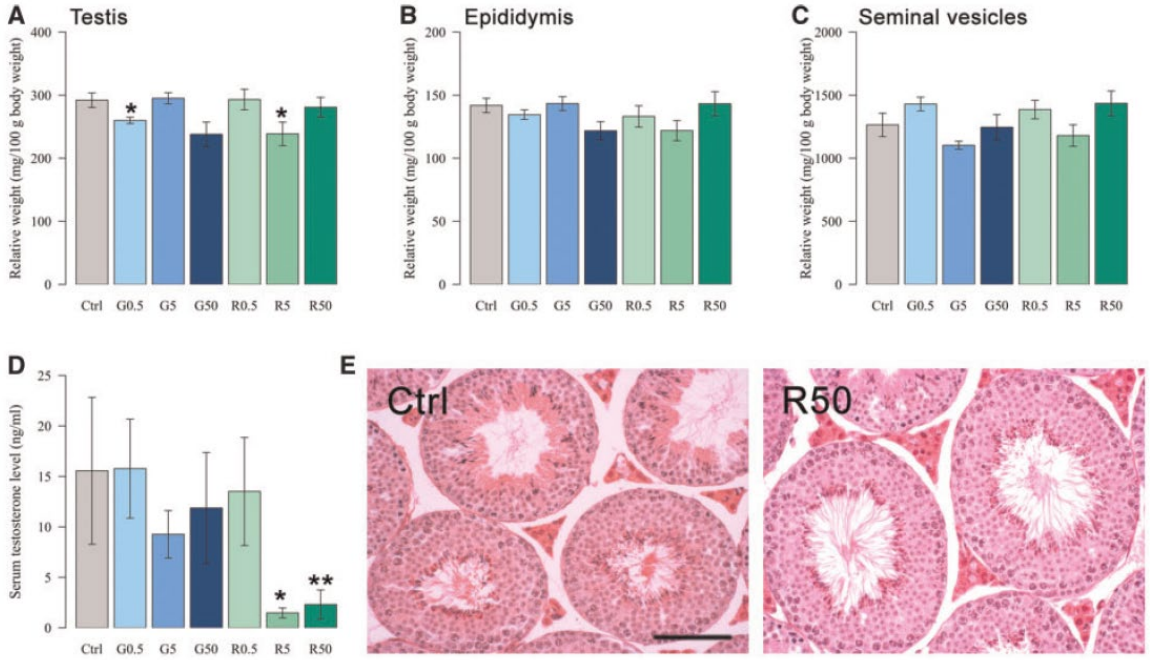

Figure 5. Perinatal exposure to glyphosate and a GBH affects the testis weight and exposure to GBH but not to glyphosate decreases serum testosterone levels in 8 m.o. mice. A, Testis; B, epididymis; and C, seminal vesicles weights in 8 m.o. mice derived from females treated with vehicle (ctrl; in gray), glyphosate (G0.5, G5, G50; in blue) and a GBH (R0.5, R5, R50; in green). Ctrl: $n = 6$; G0.5: $n = 13$; G5: $n = 20$; G50: $n = 10$; R0.5: $n = 12$; R5: $n = 6$; R50: $n = 8$ (D). The amount of testosterone level in serum were decreased in R5 and R50 groups compared with control. Values are mean ± standard error; Ctrl: $n = 12$; G0.5: $n = 13$; G5: $n = 20$; G50: $n = 10$; R0.5: $n = 12$; R5: $n = 6$; R50: $n = 7$. Statistical analyses were performed on the n number of progeny using Kruskal-Wallis test followed by Mann-Whitney post hoc test to compare the control group with ¤ treated group. $^{*}p < .05$, $^{**}p < .01$ are considered to be significantly different compared with control. E, H&E staining of the histological sections in 8 m.o. mice from ctrl and R50 experimental groups. Scale bar: 100 µm.

(Figure adapted from Pham *et al.* (2019). Perinatal Exposure to Glyphosate and a Glyphosate-Based Herbicide Affect Spermatogenesis in Mice. Toxicological Sciences, Vol. 169, Issue 1, May 2019, Pages 260–271 doi.org/10.1093/toxsci/kfz039)

Conclusion (Study report)

This study shows that glyphosate at the ADI dose of 0.5 mg/kg bw/day could have endocrine disrupting effects which could impair the male reproductive system in mice.

Assessment and conclusion

Assessment and conclusion by applicant:

The effect of glyphosate exposure from the day of vaginal plug detection to 20 days post partum via the drinking water at concentrations corresponding with 0.5, 5 and 50 mg/kg bw/day on male reproductive parameters in mice of 5, 20 and 35 days old and 8 months old was investigated. The parameters measured were the number of spermatogonia and expression of genes important to testicular function in 5–day old mice, testicular histopathology in 20–day old mice, relative weight of testes, epididymis and seminal vesicles, epididymal sperm count, serum testosterone levels, GATA1 positive cell count and ZBTB16 positive cell count in 35–day old mice, and relative weight of testes, epididymis and seminal vesicles and serum testosterone levels in 8-month old mice. No statistically significant change was found for the number of spermatogonia in 5–day old mice. The only genes of which the expression was statistically significantly changed in a dose-related fashion were Bcl2 and Kit. In 20–day old mice, sperm depleted seminiferous tubules were noted at 5 mg/kg bw/day but not at 0.5 and 50 mg/kg bw/day glyphosate. In 35–day old mice there was no statistically significant change in the relative weight of the epididymis and the seminal vesicles, epididymal sperm count and GATA1 positive cell count. No dose-effect relationship could be established for relative weight of testes, serum testosterone levels and ZBTB16 positive cell count. In 8-month old mice no statistically significant change could be observed for relative weight of epididymis and seminal vesicles and serum testosterone levels. No dose-effect relationship could be established for the decrease in relative testes weight. From these data it can be concluded that there is no evidence that glyphosate dosed orally to mice up to 50 mg/kg bw/day during the perinatal period is an endocrine disruptor and has an adverse effect on testicular function and development. This has been corroborated by reproduction toxicity studies with rats at much higher dose levels.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions

because the test conditions were not clearly described and the number of animals tested per dose level is too limited.

Reliability criteria made by the applicant

Publication: Pham *et al*., 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	N	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity ≥ 99.2 %. Source: Sigma-Aldrich.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup 3 plus.
AMPA is the tested substance		
Study		
Test species clearly and completely described	Y?	
Test conditions clearly and completely described	Y?	Not completely described. Control and treated young prepubertal or adult mice were euthanised, and reproductive organs were dissected in 5, 20, 35 days old (d.o.), and in 8 months old (m.o.) mice. Only male mice were analysed.
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	0.5, 5, 50 mg/kg bw/day.
Number of animals used per dose level reported	Y?	5 animals derived from at least 3 to 4 different litters in each group
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	Not always presented in tables.
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the test conditions were not clearly described and the number of animals tested per dose level is too limited.		

Assessment and conclusion by RMS:

In this study, the Swiss mouse were given glyphosate (G) or glyphosate-based herbicide Roundup 3 Plus (R) via drinking water at 0, 0.5, 5 and 50 mg/kg bw/day from embryonic day 0.5 to 20 days post-partum. Male offspring of the mice (at least 5 derived from 3 to 4 different litters in each group) were sacrificed at 5, 20, 35 days or 8 months for the following examinations: epididymis, seminal vesicles and testis weight, testis morphology, spermatozoa in total epididymis, serum testosterone; and the following in only G groups – number of undifferentiated spermatogonia and Sertoli cells (35 days old), expression of several genes in spermatogonia and germ cells number in testis (5 days old).

The body weight of the 20 days old mice increased in the G 0.5 group (glyphosate, 0.5 mg/kg bw/day group) and in R 5 group (Roundup, 5 mg/kg bw/day group). The body weights were not affected in the 35 days old mice but the relative testis weight decreased in the G 0.5 and R 5 groups; the epididymis weight decreased in R 0.5 group. In the 35 days old mice, the spermatozoa in total epididymis decreased in the G 5 group (-89%) and R 0.5 group (84%); and the serum testosterone decreased (by about 3 times) in G 0.5 and G 50 groups. In the 20 days old mice, there was an increase in vacuoles (all three G groups) and an increase in empty tubules with total loss of germ cells (G 0.5 group) in the seminiferous epithelium. These changes were however not noticed in the 35 days old mice and the testis morphology was normal. In the 8 months old mice, there was a decrease in relative testis weight in G 0.5 and R 5 groups and in testosterone levels in R 5 (- 90%) and R 50 (- 85%) groups.

In the 35 days old mice, the number of undifferentiated spermatogonia were decreased in the G 5 group. The expression of *Bax* (G 0.5) and *Bcl2* (all three groups) (genes involved in apoptosis); *Dazl* (G 0.5) (involved in germ cell differentiation); *Nanos3* (G 0.5 and G 50) and *Foxo1* (G 0.5) (involved in stem cell maintenance) were increased. The expression of *Kit* (G 50) and *Sall4* (all three groups) (involved in germ cell differentiation) were decreased.

Overall, there were no adverse effects with dose-response relationship (except for expression of *Bcl2*, *Kit* and *Sall4* genes) in this study. The effects on testis morphology observed with glyphosate in the 35 days old mice were not noted in the 8 months old mice. However, the exposure to Roundup decreased the relative testis weight in the mid-dose group and the testosterone levels in the mid- and high-dose groups.

The study is relevant for risk assessment as it informs on endpoints relevant to reproductive and endocrine effects. The study is however reliable with restrictions because of the following reasons: small group size, limited description of the study conditions and the results.

Category A – Ren, X. *et al.*

Data point	CA 5.6.1/018
Report author	Ren, X. *et al.*
Report year	2019
Report title	Effects of chronic glyphosate exposure to pregnant mice on hepatic lipid metabolism in offspring
Document No.	Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

The aim of the study was to investigate the toxic effects of chronic prenatal glyphosate exposure on lipid metabolism in the livers of mice fetus and offspring.

Materials and methods

Test substance	Glyphosate (provided by Shanghai Ryon Biological Technology Co., Ltd. (Shanghai, China)) and Glyphosate-based herbicide Roundup (glyphosate as isopropylamine salt) (provided by Sinochem Crop Protection Products Co., Ltd. (Shanghai, China))
Lot/Batch	Not specified
Purity/Radiochemical purity	Not specified
Animals	Ten–week-old female and male ICR mice were purchased from Nanjing Qinglongshan Experimental Animal Center (Nanjing, China). After one week of adaptation, one male and two female mice were housed in each cage from 5.00 p m. to 8.00 a m. daily to obtain pregnant mice. Pregnant mice were placed into separate cages once the pregnancy was confirmed by a vaginal smear the following morning. This day was defined as the first day of gestation. Animals were fed with water and feed *ad libitum*. The temperature and relative humidity in the animal house were controlled at 23 ± 2 °C and 50 ± 10 %, respectively, and the animals were kept on a 12-h light/dark cycle. The animal experiments were approved by the Animal Welfare Committee of Nanjing Agricultural University (Nanjing, China) and implemented in accordance with the National Institutes of Health Guidelines for Animal Care and the Committee of Animal Research Institute.
Test chemicals and preparation	Pure glyphosate (N-(phosphonomethyl)glycine) and Roundup (as the isopropylamine salt) were provided by Shanghai Ryon Biological Technology Co., Ltd. (Shanghai, China) and Sinochem Crop Protection Products Co., Ltd. (Shanghai, China), respectively. Glyphosate and Roundup were diluted with distilled water to obtain 0.5 % active ingredient solutions (w/v, 5 g glyphosate/1 L solution). Then, the subjects were administered the 0.5 % glyphosate or Roundup solution through the drinking water (pH was controlled at 7.2 ± 0.2).
Animal treatment and sampling	A total of 30 pregnant mice were randomly divided into three groups: CON (control, n = 10), GLP (0.5 % glyphosate treated, n = 10), and RU (0.5 % Roundup treated, n = 10). Half of the pregnant mice (five from each group) were exposed throughout the first 19 days of pregnancy and were sacrificed on GD19. The other half of the pregnant mice were exposed throughout the pregnancy period and given distilled water after giving birth. Weekly body weights of the offspring were recorded, and their anogenital distances were measured separately to identify their sexes. Seven and 21 days after birth, the prenatally exposed offspring were sacrificed (preferably two females and two males per mother) for blood and tissue analysis. The water consumption of the pregnant mice was measured, and the real exposure dose of glyphosate in both GLP and RU groups was approximately 7 mL (Table 1). The serum was extracted through centrifugation (3500 rpm, 15 min, 4 °C) and was used to assay the biochemical indexes. Parts of the livers were stored at 80 °C for lipid concentration determination and reverse transcriptionpolymerase chain reaction (RT-PCR). Liver samples were either fixed in a 4 % paraformaldehyde solution or embedded in optimal cutting temperature compound (O.C.T. compound) provided by Sakura Finetek Japan Co., Ltd. (Tokyo, Japan) prior to frozen sectioning for the histological observation of tissue sections.
Histological preparation	Some parts of the liver tissue were fixed in 4 % paraformaldehyde solution for 24 h and then dehydrated, clarified, embedded with paraffin and sectioned. Tissue sections (5 mm) were used for hematoxylineeosin (H&E) staining. The remaining liver tissue was embedded with O.C.T. compound and sectioned using a microtome cryostat manufactured by Thermo Fisher Scientific Instrument Co., Ltd. (Shanghai, China) for Oil Red O staining.
Serum biochemical and liver lipid concentration assays	To preliminarily diagnose the liver injury and lipid content of the organisms, the following serum biochemical indexes were determined: aspartate transaminase (AST), alanine transaminase (ALT), triglyceride (TG), total cholesterol (T-CHO), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C). Additionally, liver homogenate was centrifuged to obtain the supernatant (3500 rpm, 15 min, 4 °C) to measure the TG, T-CHO, LDL-C and HDL-C content. Both the serum biochemical indexes and hepatic lipid content were assayed with commercial reagent kits purchased from the Institute of Biological Engineering of Nanjing Jiancheng (Nanjing, China).
Analysis of gene	Total RNA was extracted from liver tissue with the ISOGEN 2 reagent kit (from

expression	NIPPON GENE CO., LTD.) (Tokyo, Japan) according to the manufacturer's instructions. The concentration of the obtained RNA was determined by a spectrophotometer, and the purity was measured using a NanoDrop® 8000. Then, PrimeScriptTM RT Master Mix (from Takara Bio Inc.) was used to reverse transcribe RNA to cDNA, which acted as a template for the SYBR® Premix Ex Taq™ PCR kit (from Takara Bio Inc.) for real-time PCR. The expression levels of the genes SREBP1C (Sterol Regulatory Element Binding Protein 1C), SREBP2 (Sterol Regulatory Element Binding Protein 2), Fasn (Fatty acid synthase, which catalyzes fatty acid synthesis), Scd (Stearoyl-CoA Desaturase 1), Acc (Acetyl-CoA Carboxylase), Hmgcr (3-hydroxy-3-methyl-glutaryl-CoA reductase), Hmgcs1 (3-hydroxy-3-methylglutaryl-CoA synthase 1), Hmgcs2 (3-hydroxy-3-methylglutaryl-CoA synthase 2) and PPARa (Peroxisome proliferator-activated receptor alpha) were determined. The relative expression levels of the above genes were normalised to b-actin expression. All primers were designed and supplied by GenScript Bio-Tech Co., Ltd. (Nanjing, China).
Data analysis	The software packages SPSS Statistics 20.0 and GraphPad Prism (GraphPad Software, San Diego, CA, USA) were utilised to analyse the data. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed. Values are expressed as the mean ± standard error of the mean (SEM), and statistical significance was set as $p<0.05$.

Results

Effects on dams - The effects of GLP and RU on pregnant mice are given in Table 1.

Table 1
Effects of chronic glyphosate exposure on the performance of pregnant mice.

Items	CON	GLP	RU	*P*
Water consumption (ml)	9.69 ± 0.76^{a}	7.88 ± 0.46^{b}	7.45 ± 0.34^{b}	0.023
Feed intake (g)	9.00 ± 0.16	8.32 ± 0.55	9.63 ± 0.72	0.261
Body weight gain (g)	32.60 ± 3.30	35.96 ± 2.92	29.84 ± 0.70	0.281
Number of fetuses (n)	10.80 ± 1.50	14.40 ± 1.57	12.60 ± 2.11	0.376
Average birth weight (g)	1.73 ± 0.13	1.65 ± 0.08	2.03 ± 0.72	0.220

Each value represents the mean ± SEM of the group (n = 5).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

(Table adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Physical and organ development - From GD19 to PND21, the body weight in both GLP and RU groups decreased and finally saw a statistically significant reduction on PND21 ($p<0.05$) (Fig. 1). When separated according to sex, offspring showed no significant differences in either body weight or weight gain among the three groups (Table 2).

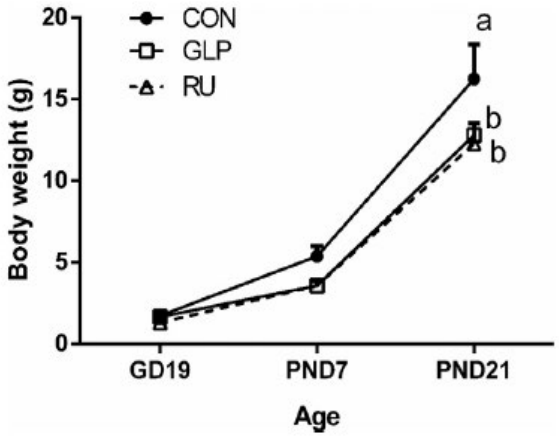

Fig. 1. Effects of chronic glyphosate exposure to pregnant mice on the body weights of offspring at the ages of GD19, PND7 and PND21 (mean ± SEM). Different letters indicate statistically significant differences, $p < 0.05$.

(Figure adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Table 2
Effects of chronic glyphosate exposure to pregnant mice on the physical development of the offspring (g).

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
Body weight	5.72 ± 0.61	4.40 ± 0.28	4.29 ± 0.59	0.429	5.24 ± 1.00	4.80 ± 0.30	4.89 ± 0.88	0.918
Body weight gain	3.19 ± 0.80	2.74 ± 0.25	3.08 ± 0.50	0.222	3.51 ± 0.88	3.15 ± 0.29	2.87 ± 0.45	0.115
PND21								
Body weight	14.69 ± 2.44	13.63 ± 0.78	13.26 ± 1.06	0.786	15.79 ± 2.21	15.10 ± 0.85	13.86 ± 1.82	0.732
Body weight gain	12.04 ± 2.01	11.98 ± 0.76	11.24 ± 0.66	0.704	14.06 ± 2.09	13.45 ± 0.82	11.84 ± 1.33	0.243

Each value represents the mean ± SEM of the group (n = 7–10).

(Table adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Liver histological observation - In both GLP and RU groups, relatively elevated numbers of vacuoles exhibiting hepatic lipid droplets were observed within the hepatocytes of both female and male offspring (Fig. 2B, C, E, H, I, K, L), when compared with the CON group. Additionally, the red areas observed in the Oil Red O stained sections represent lipid substances (Fig. 3B, C, E, H, K, L). In females, there tended to be more lipid droplets in the GLP group than in the other two groups. In contrast, in males, both the GLP and RU groups showed excessive lipid deposits. In addition, there were several clusters of monocytes in both the GLP and RU groups of PND7 females. It appears that glyphosate could cause inflammation in early-aged female mice.

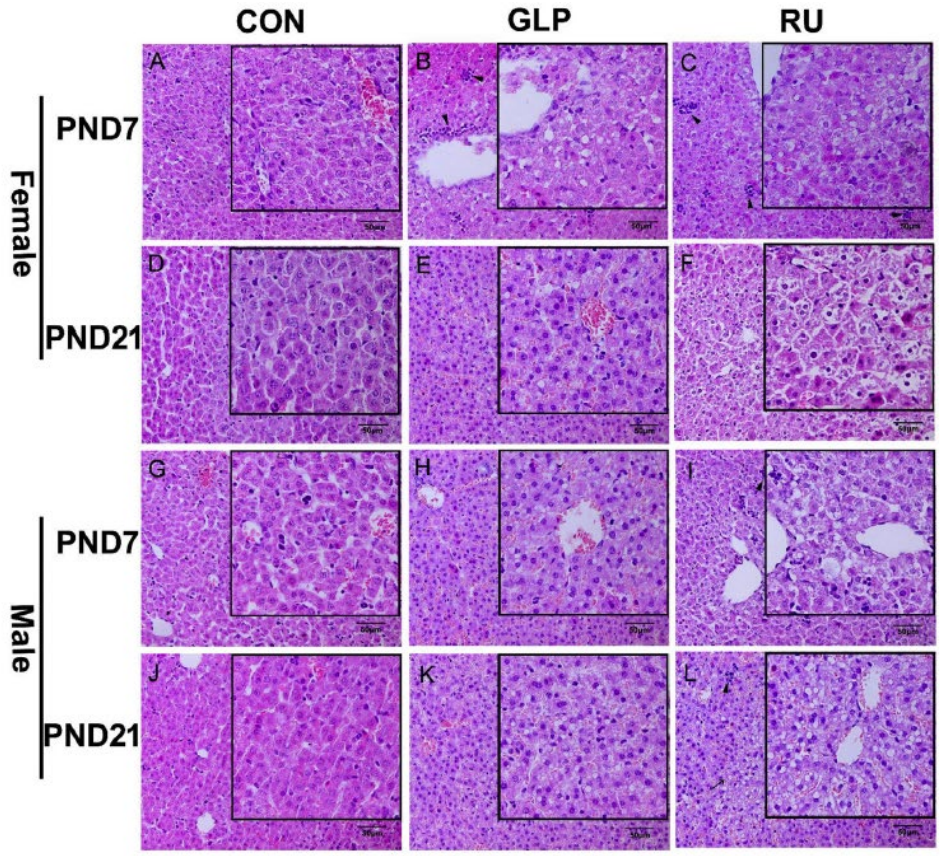

Fig. 2. Photomicrographs of H&E-stained liver sections of chronic prenatal glyphosate-treated offspring. Enlarged portions show the lipid vacuoles within the cells where the lipids have been cleared by the stain. (B), (C), (D), (H), (I) and (J) show a slightly increased number of lipid vacuoles compared with (A), (D) and (G). (K) and (L) show an obvious increase in the number of vacuoles when compared with (J). The arrowheads in (B), (C), (I) and (L) show clusters of monocytes representing inflammatory infiltration. (H&E × 400).

(Figure adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

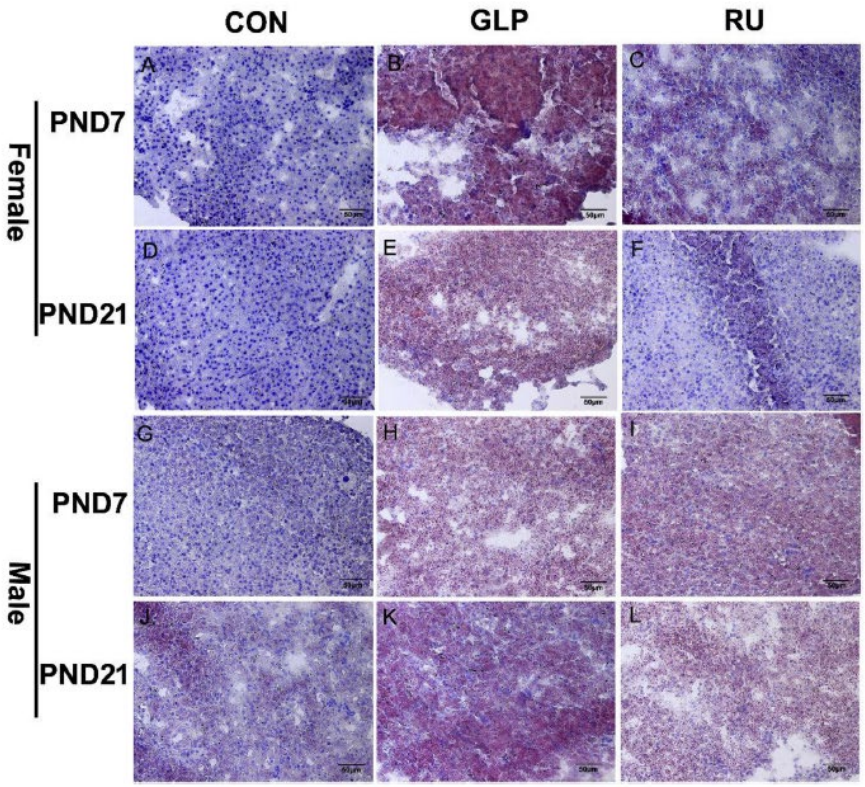

Fig. 3. Photomicrographs of liver sections stained with Oil Red O of chronic prenatal glyphosate-treated offspring. The red areas stained by Oil Red O represent the lipid deposits. (A) and (D) show the normal liver tissue of the female offspring. (B) and (E) show substantial lipid deposits from the GLP group, while there are slight lipid deposits in (C) and (F) from the RU group. (G) and (J) show the normal liver tissue with few lipid substances in the CON group. (H), (I) and (K) show more lipid deposits than do (G) and (J). (Oil-red O × 400). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Figure adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Serum biochemical index - Compared with the CON group, TG levels showed a significant increase in the GLP group in both GD19 foetuses ($p<0.01$) (Table 3) and PND21 female mice ($p<0.05$) (Table 4). With respect to T-CHO levels, GLP mice showed a remarkable increase in both PND7 males ($p<0.01$) and PND21 females ($p<0.05$) compared with CON mice. LDL-C levels also increased in PND7 mice in both the GLP and RU groups ($p<0.05$) (Table 4). The increased lipid content reflects the adverse effects of glyphosate on lipid metabolism, although this disturbed effect was not detected in every individual. Furthermore, significantly elevated AST levels in PND7 females in the RU group ($p<0.01$) are theoretically considered to be a result of an injured liver.

Table 3
Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes in fetuses.

Items	CON	GLP	RU	*P*
TG (mmol/L)	0.15 ± 0.10^{b}	0.79 ± 0.21^{a}	0.30 ± 0.10^{b}	0.002
T-CHO(mmol/L)	2.15 ± 0.49	2.56 ± 0.52	1.22 ± 0.06	0.165
LDL-C (mmol/L)	1.03 ± 0.09	1.08 ± 0.04	0.91 ± 0.05	0.185
HDL-C (mmol/L)	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.01	0.433
AST (IU/L)	67.88 ± 14.17	103.20 ± 10.30	76.21 ± 10.55	0.134
ALT (IU/L)	20.71 ± 1.02	47.54 ± 10.20	32.09 ± 2.35	0.091

Each value represents the mean ± SEM of the group ($n = 20$).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

(Table adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Table 4
Effects of chronic glyphosate exposure to pregnant mice on the blood biochemical indexes of PND7 and PND21 offspring.

Items	Female				Male			
	CON	GLP	RU	*P*	CON	GLP	RU	*P*
PND7								
TG (mmol/L)	1.46 ± 0.07	1.79 ± 0.26	1.60 ± 0.08	0.324	1.11 ± 0.14	1.45 ± 0.14	1.45 ± 0.20	0.240
T-CHO (mmol/L)	1.46 ± 0.07	1.60 ± 0.27	1.42 ± 0.19	0.792	2.01 ± 0.23^{b}	2.92 ± 0.19^{a}	1.97 ± 0.11^{b}	0.007
LDL-C (mmol/L)	1.30 ± 0.18^{b}	1.73 ± 0.07^{a}	1.42 ± 0.07^{ab}	0.047	0.58 ± 0.07^{c}	0.99 ± 0.12^{b}	1.75 ± 0.11^{a}	0.000
HDL-C (mmol/L)	1.12 ± 0.11	1.18 ± 0.09	0.86 ± 0.04	0.074	0.86 ± 0.12	0.88 ± 0.04	1.13 ± 0.11	0.189
AST (IU/L)	28.03 ± 6.14^{b}	29.09 ± 6.22^{b}	63.84 ± 8.03^{a}	0.008	65.17 ± 7.65	72.54 ± 10.41	81.71 ± 10.52	0.503
ALT (IU/L)	30.93 ± 3.77	40.51 ± 4.86	39.89 ± 4.08	0.245	29.22 ± 2.39	32.01 ± 2.89	39.27 ± 2.29	0.058
PND21								
TG (mmol/L)	1.30 ± 0.18^{b}	2.41 ± 0.37^{a}	1.36 ± 0.15^{ab}	0.022	1.01 ± 0.11	1.10 ± 0.37	1.19 ± 0.38	0.741
T-CHO (mmol/L)	1.30 ± 0.18^{b}	2.06 ± 0.16^{a}	1.37 ± 0.15^{b}	0.028	0.92 ± 0.07	1.01 ± 0.06	1.05 ± 0.21	0.778
LDL-C (mmol/L)	2.26 ± 0.26	2.23 ± 0.12	2.65 ± 0.18	0.293	1.24 ± 0.19	1.08 ± 0.27	0.64 ± 0.13	0.115
HDL-C (mmol/L)	1.41 ± 0.28	1.65 ± 0.31	1.29 ± 0.10	0.570	1.56 ± 0.31	1.49 ± 0.17	1.22 ± 0.29	0.713
AST (IU/L)	41.14 ± 12.38	70.64 ± 9.87	50.04 ± 12.01	0.286	39.37 ± 9.28	54.06 ± 8.10	22.56 ± 8.90	0.091
ALT (IU/L)	19.02 ± 0.43	22.50 ± 4.61	19.48 ± 3.20	0.722	14.27 ± 4.06	16.01 ± 2.34	16.34 ± 3.04	0.890

Each value represents the mean ± SEM of the group (n = 7–10).
Different letters indicate statistically significant differences. a, b $p < 0.05$.

(Table adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Lipid concentration in the liver - Compared with that in CON mice, TG levels in the RU group significantly increased in GD19 foetuses and PND7 female offspring ($p<0.05$) (Fig. 4A and B). Moreover, T-CHO levels of both PND7 and PND21 offspring increased in the GLP or RU groups ($p<0.05$) (Fig. 4B-E). Elevated TG and T-CHO levels in the liver can probably cause lipid deposits. The LDL-C levels of PND21 males showed a noticeable increase in both the GLP and RU groups ($p<0.05$) (Fig. 4E), and the HDL-C levels in PND7 males were elevated in the RU group ($p<0.05$) (Fig. 4C). Both low-density and high-density lipoproteins can transport cholesterol in the extracellular environment. The elevated level of these proteins in serum is considered to be the result of increased cholesterol levels.

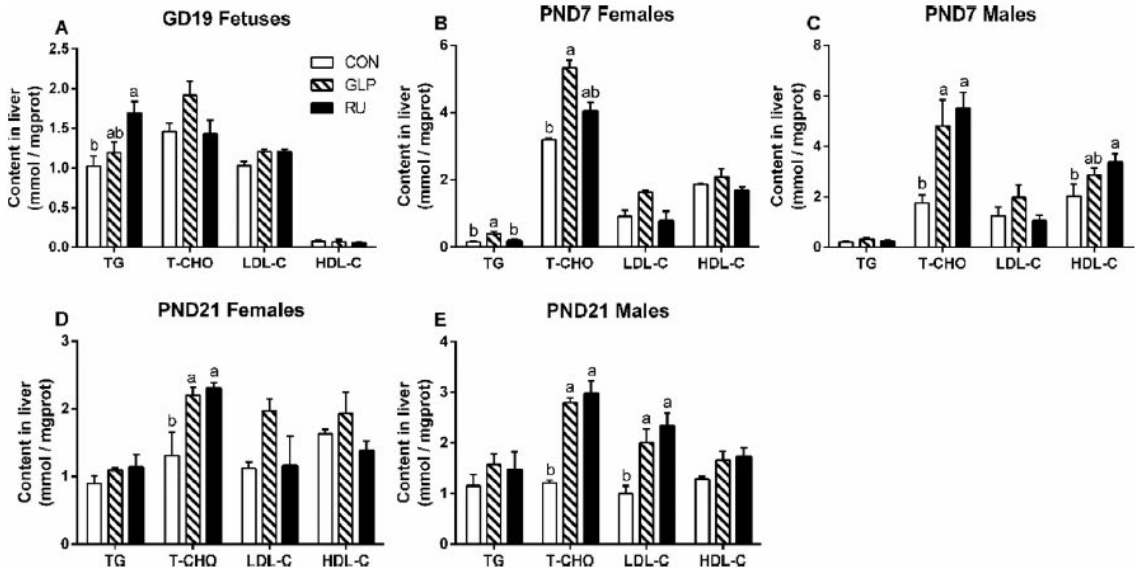

Fig. 4. Effects of chronic prenatal glyphosate exposure on the lipid content in the livers of the offspring (mean ± SEM). (A) shows the TG, T-CHO, LDL-C and HDL-C content in livers of GD19 fetuses, and (B), (C), (D) and (E) show these parameters in PND7 and PND21 females and males, respectively. Different letters indicate statistically significant differences. a, b $p < 0.05$.

(Figure adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Expression levels of genes related to lipid metabolism in the liver - The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1 and Hmgcs2 in the GLP and RU groups showed a significant increase in GD19 foetuses and PND7 and PND21 offspring ($p<0.05$) (Fig. 5). These genes are closely related to hepatic lipid production, so their elevation contributes to increased fat storage. However, this kind of increase does not match well to the trend in serum lipid content alteration. The levels of PPARα in PND7 males and PND21 females increased remarkably in both the GLP and RU groups, which is likely due to the growing demand for lipid catabolism caused by the increased lipid content.

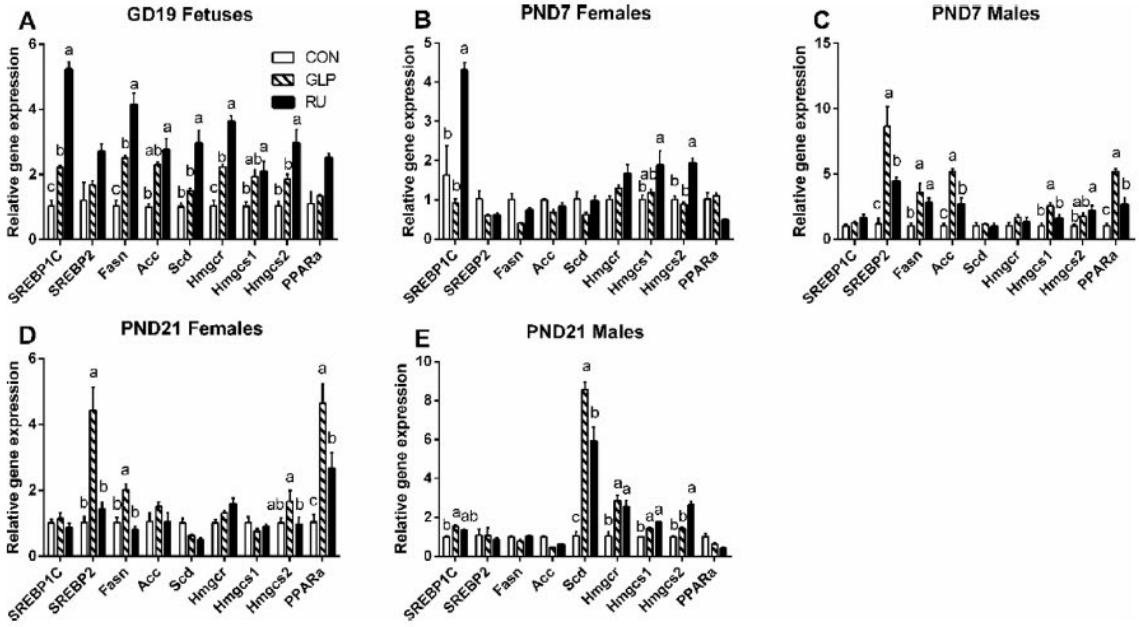

Fig. 5. Effects of chronic prenatal glyphosate exposure on relative mRNA expression levels in the livers of the offspring (mean ± SEM). The relative expression levels of the genes SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1, Hmgcs2 and PPARα genes in the livers of (A) fetuses, (B) PND7 female offspring, (C) PND7 male offspring, (D) PND21 female offspring, and (E) PND21 male offspring are shown. Different letters indicate statistically significant differences. a, b $p < 0.05$.

(Figure adapted from Ren *et al.* (2019). Effects of chronic glyphosate exposure to pregnant mice on hepatic Environmental Pollution 254 (2019) 112906 doi.org/10.1016/j.envpol.2019.07.074)

Conclusion

Chronic prenatal glyphosate exposure can probably cause lipid metabolism disruption in offspring, accompanied by an elevated lipid content in both serum and liver tissue. These alterations in hepatic lipid metabolism might result from rising lipogenesis in hepatocytes through increasing related gene expression.

Assessment and conclusion

Assessment and conclusion by applicant:

The current study aimed to examine any effects on lipid metabolism in foetuses and pups following prenatal exposure to glyphosate or the glyphosate formulation, Roundup™. Ten pregnant female rats per group were exposed from gestation day 1 through 19 to drinking water containing either 0.5 % glyphosate, prepared using "pure" glyphosate (N-(phosphonomethyl) glycine), or 0.5 % glyphosate using an appropriate dilution of Roundup™. A similar group of animals were given distilled water and served as the control group. Five females per group were terminated on gestation day 19 for examination of foetuses, while the remaining dams were allowed to litter and maintain their litters to postnatal day 21. Offspring (2/sex/litter where possible) were selected on postnatal days 7 and 21 for evaluation. Foetal and offspring evaluations included liver histology, serum biochemistry, liver lipid concentration and gene expression analysis of genes related to lipid metabolism in the liver.

The study is non-GLP and does not report the following information;

- Purity of test items
- Body weight and clinical signs for pregnant animals
- Clinical observations of offspring
- Achieved dose of glyphosate in treated animals in mg/kg bw/day.
- Measures to control inter animal and intergroup variability such as
- Time of necropsy distributed equally across groups
- Standardisation of litter size on day 4 of lactation to mitigate variability caused by differences in liter size.
- More than a single dose level of glyphosate (0.5 % solution); thus, preventing dose-response characterisation
- Liver weight of foetuses or offspring
- Normal physiological ranges for serum and liver biochemistry in this strain of rat at this laboratory
- Clear reporting of statistical evaluation and differences

- Thorough histological evaluations of the liver with incidence and severity of any recorded findings.

Although the authors concluded that there were treatment-related effects on foetal and offspring body weight, there is no evidence from this study to suggest that glyphosate exposure has had any impact on foetal development or pup development postnatally. There was no effect on average birth weight of pups, the slight difference observed in the glyphosate-treated group should be attributed to the slightly larger mean litter size observed (14.4 pups compared to 10.8 in the control group). Although figure 1 shows a reduction in mean pup weight in the glyphosate and Roundup™ treated groups (male and females combined). Group mean body weight of pups by sex showed no statistical differences from control.

Given the small group size, the large inter animal variability observed, the lack of consistency between the same parameter across the sexes, timepoints or sampling matrices, and the deficiencies listed above, it is not possible to clearly attribute any of the observed differences to glyphosate exposure. Therefore, the current study provides no evidence that glyphosate exposure causes lipid metabolism disruption in offspring following prenatal (in utero) exposure.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterised, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.

Reliability criteria made by the applicant

Publication: Ren *et al.,* 2019	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	N	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Shanghai Ryon Biological Technology Co, Shanghai, China.
Only glyphosate acid or one of its salts is the tested substance	N	Also formulation was tested: Roundup from Sinochem Crop Protection Products, Shanghai, China.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	?	Mice.
Test conditions clearly and completely described	?	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	One dose level: 0.5 % in drinking water.
Number of animals used per dose level reported	Y	10/dose.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate used is not sufficiently characterised, only one dose level was tested, there was large inter animal variability observed and too few animals per dose level were analysed.

Assessment and conclusion by RMS:
In this study, 10 ICR mice/group were given glyphosate (G) or glyphosate-based herbicide Roundup (R) via drinking water at a single dose level of 0.5% of glyphosate in both groups from gestation day (GD) 1 to GD19. A drinking water concentration of 0.5% (5g glyphosate/L) will corresponds to 1000 mg/kg bw/day using a default value of 0.2 for subacute studies according to EFSA guidance document on default values (EFSA Journal 2012;10(3):2579). A similar group of animals were given distilled water and served as the control group. Five dams/group were sacrificed on GD19 and fetuses were examined. The liver and serum samples of the fetus/offspring (2/sex/litter preferred; on PND7 and PND21) were collected to examine the following: the serum biochemical indexes, histopathological observations, lipid concentrations and mRNA gene expression levels that are related to lipogenesis and lipid catabolism in the livers.

There was a statistically significant decrease in body weight gain of the offspring on PND21 in both G and R groups. There was no difference though when the values were considered on the basis of sex. Liver histopathology showed increased vacuoles with lipid droplets (more in females of the G group), more red areas representing lipid substances and clusters of monocytes (PND7 females in both G and R groups). Following changes were observed in the serum biochemistry: increased triglycerides in GD19 fetuses and PND21 females of the G group; increased total cholesterol in PND7 males and PND21 females of the G group; and increased aspartate transaminase in PND7 females of the R group. Following changes were observed in the lipid concentration in the liver: increased triglycerides in GD19 fetuses and PND7 females of the R group; increased total cholesterol in PND7 and PND21 offspring in both the G and R groups; increased low-density lipoprotein cholesterol in PND21 males of both the G and R groups; and increased high-density lipoprotein cholesterol in PND7 males of the R group. Following changes were observed in the expression of genes related to lipid metabolism in the liver: increased relative expression of SREBP1C, SREBP2, Fasn, Acc, Scd, Hmgcr, Hmgcs1 and Hmgcs2 in GD19 fetuses, PND7 and PND21 offspring of both the G and R groups; and increased expression of PPARα in PND7 males and PND21 females of both the G and R groups.

The results of the study show some changes in lipid metabolism in the offspring exposed prenatally, however the clinical relevance of this finding is lacking and no firm conclusion can be made due to the uncertainties including low number of animals examined and individual variability. The study is relevant for risk assessment as it informs on endpoints relevant to adverse effects in offspring exposed prenatally. The study is however reliable with restrictions because of the deficiencies as listed by the Applicant.

Category A – Zhang, J.W. *et al.*

Data point	CA 5.6.1/019
Report author	Zhang, J.W. *et al.*
Report year	2019
Report title	The toxic effects and possible mechanisms of glyphosate on mouse oocytes
Document No.	Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study
The aim of the study was to investigate whether glyphosate exposure would affect the developmental ability of mouse oocytes *in vitro*.

Materials and methods

Test substance	Glyphosate (source not specified)
Lot/Batch	Not specified.
Purity/Radiochemical purity	Not specified.
Antibodies and chemicals	Rabbit polyclonal anti-LC3A/B (light chain 3, LC3), anti-p-MAPK, anti-γ-H2AX and anti-β-actin antibodies were purchased from Cell Signaling Technology. Mouse monoclonal anti-Bax, anti-Bcl-2, antiAtg12, anti-Annexin V and anti-α-tubulin antibodies were purchased from Santa Cruz Biotechnology. The Reactive Oxygen Species Assay Kit was purchased from Beyotime Biotechnology (S0033). The Mitochondrial Membrane Potential Assay Kit with JC-1 was obtained from Beyotime (C2005). Mito Tracker Red CMXRos was purchased from Cell Signaling Technology (#9082).
Mice	Female Kunming mice (25-30 g) were purchased from the Institute of Zoology, Chinese Academy of Sciences. They were housed in a temperature-controlled room with 12D:12L (dark vs. light) and had unrestricted access to food and water under conditions of constant temperature (23 ± 2° C).
Oocyte collection and treatment	To collect fully grown germinal vesicle (GV) oocytes, the mice were superovulated by injecting them intraperitoneally with 10 IU Pregnant Mares Serum Gonadotropin (PMSG) 48 h earlier. The mice were sacrificed by cervical dislocation and the ovaries were placed in M2 medium. Oocytes were released from the ovaries by puncturing the follicles with a fine needle, and denuded oocytes were collected by gentle pipetting. GV oocytes were cultured in an incubator in 50 μL droplets of culture medium under liquid paraffin oil at 37 °C in 5 % CO_2. Glyphosate was dissolved in M16 medium and diluted to a final concentration of 50, 100, 200 or 500 μM. After culturing for 2 h or 14 h, germinal vesicle breakdown (GVBD) and metaphase II (MII) oocytes were used for the subsequent experiments.
Immunofluorescent staining	To determine the levels of intracellular reactive oxygen species (ROS) production, denuded MII oocytes from 6 mice were incubated with M16 medium that contained 10 μM dichlorofluorescein diacetate (DCFH-DA) for 30 min at 37 °C in the dark. After washing three times with 1 % Bovine Serum Albumin/Phosphate-buffered saline (BSA/PBS), the oocytes were placed in 50 μL of M16 medium droplets and the fluorescence was observed with a confocal laser-scanning microscope (Zeiss LSM 710 META, Germany) with the same scanning parameters. The fluorescence intensity of each oocyte was analysed using ImageJ software. For mitochondrial staining, MII oocytes from 6 mice were fixed in 4 % paraformaldehyde (PFA) for 1 h and then placed in membrane permeabilisation solution containing 0.5 % Triton X-100 for 20 min at room temperature. After washing three times with 1 % BSA/PBS, the oocytes were incubated in 200 nM Mito Tracker Red CMXRos in M16 medium for 30 min in the dark. After several washes, the oocytes were costained with DAPI (4', 6-diamidino-2- phenylindole) for 5 min. Images were captured by the confocal laser-scanning microscope. To measure mitochondrial membrane potential (MMP), MII oocytes from 6 mice were incubated at 37 °C for 20 min with the 1 x JC-1 probe, then washed twice with JC-1 buffer (5 min each) to remove surface fluorescence. Images of fluorescence were captured using confocal microscopy as above. MMP was quantified as the ratio of red to green fluorescence using ImageJ software. To measure the spindle, apoptosis, autophagy and DNA damage, MII oocytes from 24 mice were stained with anti-α-tubulin, antiAnnexin-V, anti-LC3and anti-γ-H2AX antibodies, respectively. The denuded MII oocytes were fixed with 4 % PFA for 30 min, permeabilised in 0.5 % Triton-X 100 for 20 min at room temperature, and blocked with 1 % BSA/PBS for 1 h at 4 °C. The oocytes were incubated with anti-α-tubulin (1:200), anti-LC3 A/B (1:200), antiAnnexin-V (1:200) and anti-γ-H2AX (1:200) at room temperature for 2 h followed by three washes with 1 % BSA/PBS. The oocytes were then incubated with an appropriate secondary antibody for 2 h at room temperature and then costained with DAPI for 5 min in the dark. After three washes, oocytes were mounted on a glass slide and representative images were captured by the confocal laser-scanning microscope. ImageJ software was used to quantify the fluorescence intensity in the images.

Quantitative real-time PCR	Total RNA was extracted from 100 MII oocytes from 12 mice using the TRNpure Total RNA Kit (Nobelab, Beijing, China) according to the manufacturer's instructions. The first strand cDNA was generated with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, China). All gene expression was determined using the FS Universal SYBR green PCR master mix (Roche, Canada) under the following conditions: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each sample was tested in triplicate and gapdh was used as an internal control gene. Relative expression levels were analysed by the 2-ΔΔCt method.
Western blot	A total of 200 MII oocytes from 24 mice were lysed in 1 x SDS sample buffer containing 1 x protease inhibitor Cocktail (cwbiotech) on ice for 20 min. They were boiled for 5 min at 95 °C, then subjected to 12 % SDS-PAGE and the separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked for 2 h with 5 % nonfat milk in TBST and then probed with primary antibodies for 2 h at room temperature (anti-p-MAPK antibody, 1:500; anti-Bcl-2 antibody, 1:1000; anti-Bax antibody, 1:1000; anti-LC3 antibody, 1:1000; anti-Atg12 antibody, 1:500; anti-α-tubulin antibody, 1:1000; and anti-β-actin antibody, 1:1000). After washing three times with TBST (10 min each), membranes were incubated for 1 h with the appropriate HRP-conjugated secondary antibodies (1:1000). Three washes later, the protein bands were visualised using BeyoECL Plus (Thermo Fisher Scientific), and the signals were acquired by Tanon 5500. The images were quantified using ImageJ software.
Statistical analysis	At least 50 oocytes were analysed for each experiment. For each treatment, at least three biological replicates were performed and data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were performed using the GraphPad Prism software followed by Student's unpaired two-tailed t-test and two-way ANOVA. Statistical significance was set at P value: *<0.05, **<0.001, ***<0.0005, ****<0.0001.

Results

Glyphosate composition analysis - The chemical composition of glyphosate was analysed before the start of the experiment by Ultra Performance Liquid Chromatography/Quadrupole-Time-of-Flight-Mass Spectrometry (UPLC/Q-TOF-MS) (Fig. 1). Specific results from this analysis were not reported.

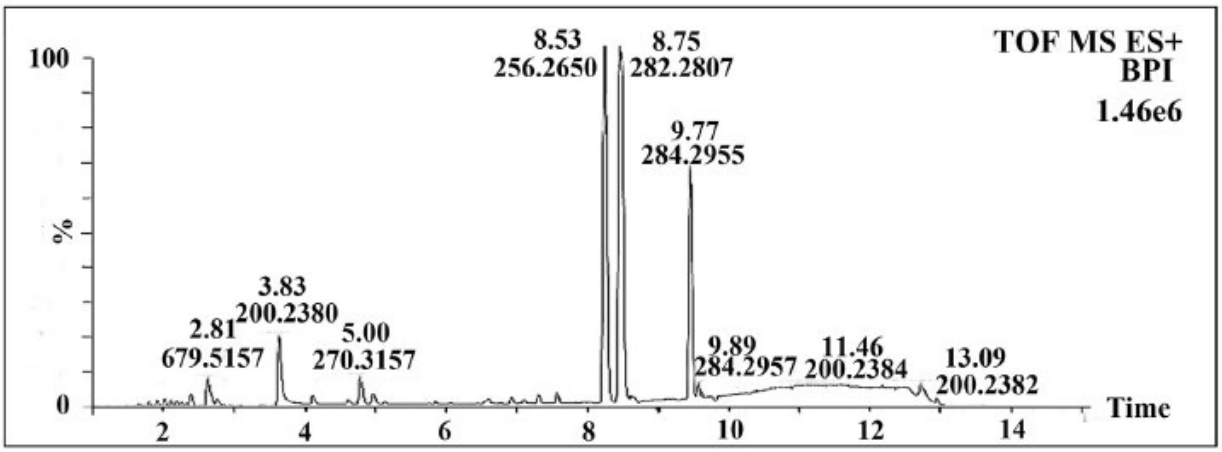

Fig. 1. Analysis of glyphosate chemical constituents by UPLC-Q-TQF-MS (adapted from Zhang, J.W. *et al.* 2019).
(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Glyphosate treatment reduced the GVBD and polar body extrusion (PBE) in mouse oocytes - To test the effect of glyphosate on the mouse oocytes maturation, the oocytes were cultured in culture medium supplemented with increasing concentrations of glyphosate (50, 100, 200 or 500 μM). Typical images of exposure to glyphosate on GVBD and polar body extrusion (PBE) are shown in Fig. 2. After treatment with glyphosate, GVBD (Fig. 2A and B) and PBE (Fig. 2C and D) were significantly decreased in the 200 mM and 500 mM groups, while there was no significant variation in the 50 μM and 100 μM treatment groups. Hence, the 500 μM glyphosate treatment was adopted for subsequent experiments. These observations were considered to show that glyphosate exposure decreased mouse oocyte developmental competence.

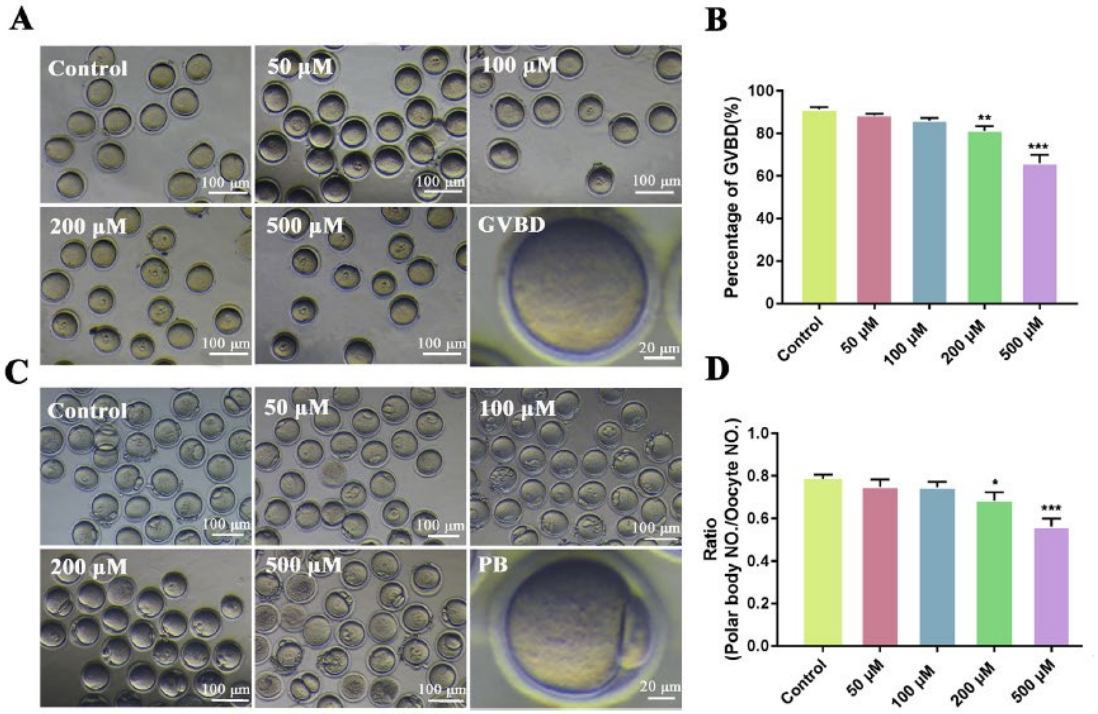

Fig. 2. Effects of glyphosate on GVBD and PBE in mouse oocytes. (A) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 2 h. (B) GVBD rates of different groups after 2 h of treatment. The data are expressed as the mean ± SEM. (C) Representative images of mouse oocytes after exposure to different concentrations of glyphosate for 14 h. (D) Quantification of the presence of PBE in control and treatment oocytes. The data are expressed as the mean ± SEM.

(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Glyphosate treatment increased ROS generation and DNA damage in mouse oocytes - It was tested whether defects observed in glyphosate-exposed mouse oocytes were mediated by oxidative stress. As shown in Fig. 3A and B, the fluorescence intensity of DCFH-DA was significantly higher in the 500 μM glyphosate treatment group than in the control oocytes. The levels of sod2 and gpx mRNA expression were significantly increased in glyphosate-exposed oocytes when compared with the control group (Control: sod2: 1.005 ± 0.066, gpx: 1.003 ± 0.052; 500 μM: sod2: 1.702 ± 0.148, gpx: 1.545 ± 0.181); the expression of cat mRNA was also increased (Control: 1.001 ± 0.036; 500 μM: 1.241 ± 0.111). These results suggested that glyphosate administration enhanced ROS production. As described above, glyphosate is cytotoxic to oocytes. As shown in Fig. 3D, there were no significant double-strand breaks (DSB) foci in the control oocytes; however, there was a dense mass of γ-H2AX foci associated with chromatin in glyphosate-exposed oocytes. As shown in Fig. 3E, the fluorescence intensity of γ-H2AX also significantly increased after glyphosate treatment compared to that in the control group. These results were considered to indicate that glyphosate treatment results in DNA damage in mouse oocytes.

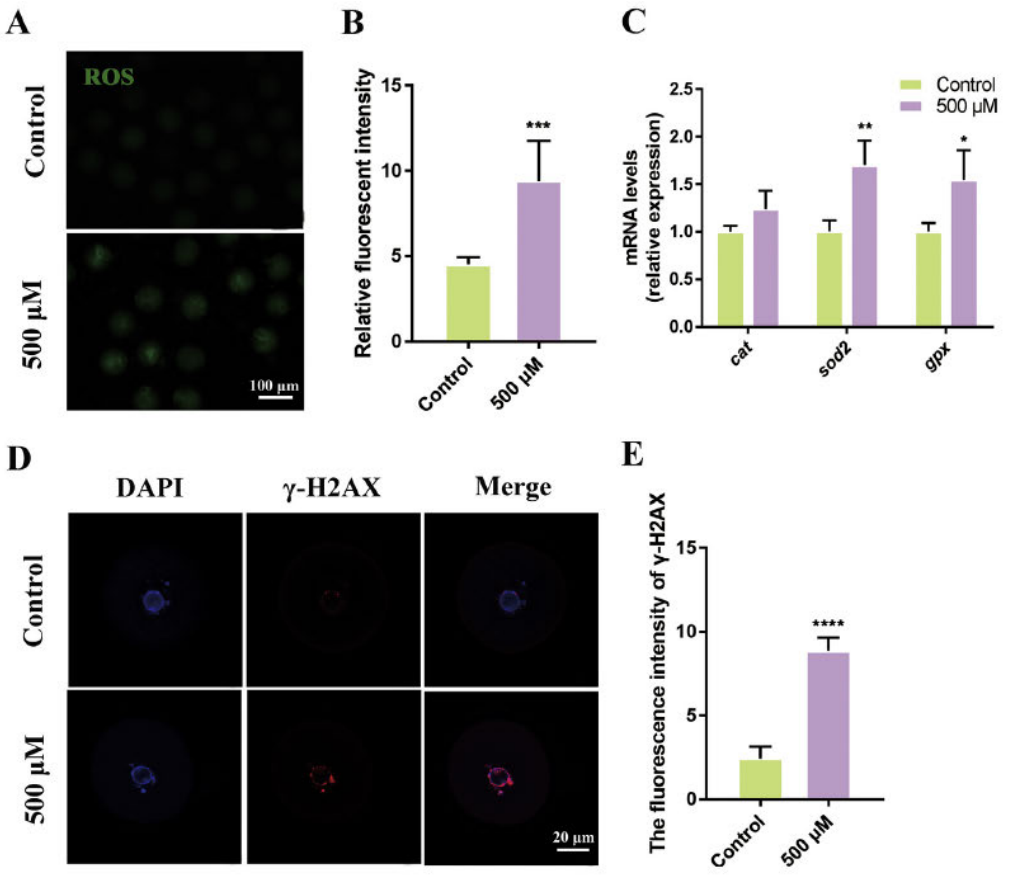

Fig. 3. Effects of glyphosate on ROS generation and DNA damage in mouse oocytes. (A) Representative images of DCFH-DA fluorescence in control and glyphosate-exposed mouse oocytes. (B) Quantitative analysis of ROS fluorescence intensity in control and glyphosate-exposed groups. The data are expressed as the mean ± SEM. (C) Relative expression levels of oxidative stress-related genes were examined by qRT-PCR. The data are expressed as the mean ± SEM. (D) Representative images of control and glyphosate-treated oocytes exhibiting γ-H2AX immunostaining. (E) The γ-H2AX fluorescence intensity obviously increased after glyphosate treatment. The data are presented as the mean ± SEM.

(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Glyphosate treatment disturbs spindle morphology in mouse oocytes - To investigate whether the oocytes with glyphosate treatment showed defects in spindle positioning and chromosome scattering, MII oocytes were assessed by immunocytochemical staining with anti-α-tubulin antibody and DAPI. In contrast to oocytes from the control group, which presented a normal spindle appearance and well-aligned chromosomes at the equatorial plate, the glyphosate-exposed group showed misaligned chromosomes and abnormal spindle morphology (Fig. 4A). The rate of abnormal spindles in the glyphosate treatment group was also increased (Control: 9.333 ± 0.491%; 500 μM: 15.767 ± 1.369%). as shown in Fig. 4B. The effect of glyphosate on the expression levels of spindle assembly regulatory protein was also assessed. In Fig. 4C and D, Western blots revealed that the level of p-MAPK protein was significantly reduced after 500 μM glyphosate treatment (Control: 0.856 ± 0.053; 500 μM: 0.517 ± 0.070). These results were considered to suggest that glyphosate treatment disturbs spindle morphology in mouse oocytes.

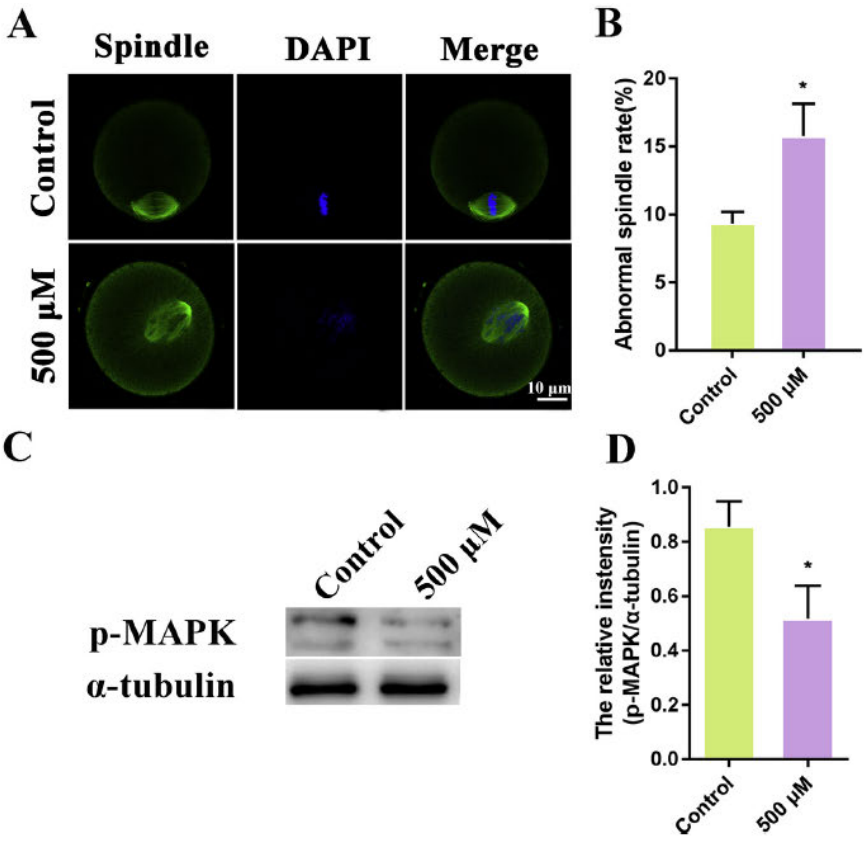

Fig. 4. Effects of glyphosate on spindle morphology and chromosome alignment in mouse oocytes. (A) Representative confocal images of spindle morphology in control and glyphosate exposed groups. (B) Percentage of cells exhibiting abnormal spindle/chromosome morphology. The data are expressed as the mean ± SEM. (C) The expression level of p-MAPK protein in oocytes from different groups was analysed by Western blot analysis. (D) The relative intensity of p-MAPK protein expression (p-MAPK/α-tubulin) was significantly reduced after treatment with 500 μM glyphosate. The data are expressed as the mean ± SEM.

(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Glyphosate treatment resulted in mitochondrial injury in mouse oocytes - A homogeneous distribution of mitochondria was present in the control group, but an aggregated distribution was seen in the glyphosate-exposed group by staining with Mito Tracker Red (Fig. 5A). Alterations in MMP of mouse oocytes from different groups were evaluated by staining with JC-1 (Fig. 5B). As shown in Fig. 5C, the result illustrated that the MMP was significantly lower in the glyphosate-exposed group compared with the control group (Control: 1.871 ± 0.082; 500 μM: 4.517 ± 0.155). Based on these data, glyphosate treatment was considered to interfere with mitochondrial function in mouse oocytes.

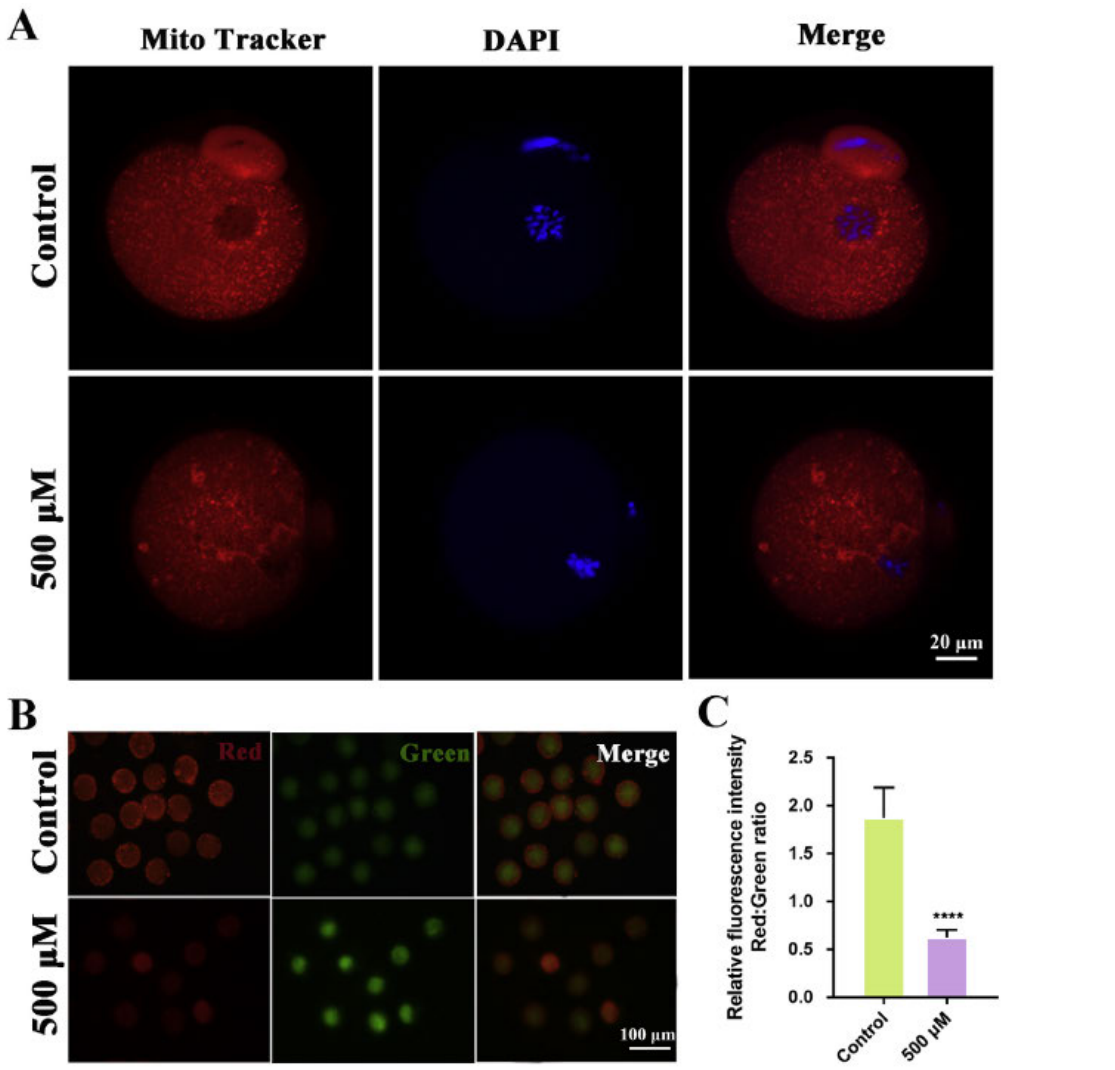

Fig 5. Glyphosate treatment results in mitochondrial injury in mouse oocytes. (A) Representative images of mitochondrial distribution in control and glyphosate-treated oocytes by staining with MitoTracker Red. (B) Representative images of MMP in mouse oocytes from different groups stained with JC-1. (C) MMP levels (red/green fluorescence intensity) in glyphosate-exposed oocytes was significantly lower than that in the control group. The data are presented as the mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Glyphosate treatment induced early apoptosis and autophagy in mouse oocytes - Annexin-V staining was conducted to identify whether early apoptosis occurred in glyphosate-treated oocytes. In the control group, Annexin-V signals were detected only in the zona pellucida, whereas the treatment group had a clear green signal in the membrane and zona pellucida (Fig. 6A). In Fig. 6B, the apoptotic fluorescence intensity of oocytes was notably higher in the treatment group than in the controls. Apoptosis-related protein expression levels were also assayed via As shown in Fig. 6G and H, Western blot analysis, which showed that the expression of Bcl-2 protein decreased and the expression of Bax protein increased after exposure to 500 μM glyphosate (Control: Bcl-2: 0.645 ± 0.009, Bax: 0.427 ± 0.020; 500 μM: Bcl-2: 0.549 ± 0.011, Bax: 0.556 ± 0.018). To confirm this result, the mRNA expression levels of apoptosis-related genes were tested by qRT-PCR. The qRT-PCR results were consistent with the Western blot results. Together, these results were considered to indicate that glyphosate induced early apoptosis in mouse oocytes. Whether autophagy had occurred in glyphosate-treated oocytes was assessed by LC3 immunofluorescent staining; As shown in Fig. 6D and E, the fluorescence intensity was significantly higher in the 500 μM glyphosate treatment group than in the oocytes from the control group. The mRNA expression levels of autophagy-related genes were also assessed by qRT-PCR and showed an increasing trend (Control: lc3: 0.860 ± 0.013, atg14: 1.024 ± 0.149, mtor: 0.847 ± 0.171; 500 μM: lc3: 1.591 ± 0.109, atg14: 1.800 ± 0.139, mtor: 1.268 ± 0.242) (Fig. 6F). Western blot analysis showed that the expression of LC3 and Atg12 protein increased after exposure to 500 μM glyphosate (Control: LC3: 0.280 ± 0.021, Atg12: 0.435 ±

0.007; 500 µM: LC3:0.504 ± 0.030, Atg12: 0.580 ± 0.016) (Fig. 6I and J). Together, these results were considered to indicate that glyphosate induced autophagy in mouse oocytes.

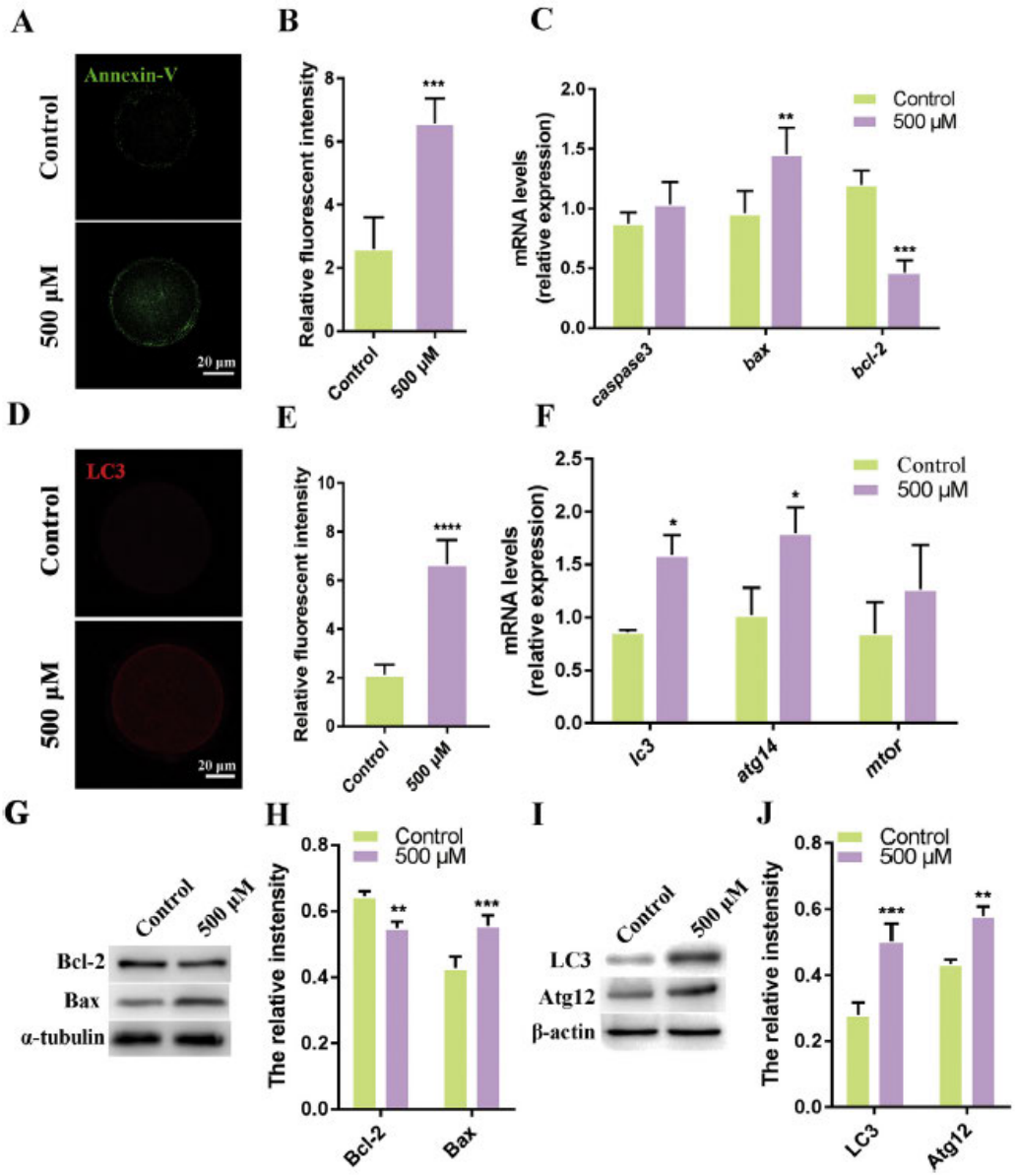

Fig. 6. Glyphosate treatment induces early apoptosis and autophagy in mouse oocytes. (A) Representative images of apoptosis (Annexin-V staining) in mouse oocytes. (B) Quantification of Annexin-V signal was recorded in control and glyphosate-exposed oocytes. (C) Relative mRNA levels of *caspase3*, *bax* and *bcl-2* in different groups of oocytes. (D) Representative images of autophagy (LC3 staining) in mouse oocytes. (E) Quantification of the fluorescence signal was recorded for the control and glyphosate-exposed oocytes. (F) The relative mRNA level of *lc3*, *atg14* and *mtor* in different groups oocytes. (G) The expression levels of apoptosis-related protein in oocytes from different groups were analysed by Western blot analysis. (H) The relative intensities of Bcl-2 and Bax protein expression were calculated. (I) The expression levels of autophagy-related proteins in oocytes from different groups were analysed by Western blot analysis. (J) The relative intensities of LC3 and Atg12 protein expression were calculated.

(Figure adapted from Zhang *et al.* (2019). The toxic effects and possible mechanisms of glyphosate on mouse Oocytes. Chemosphere 237 (2019) 124435 doi.org/10.1016/j.chemosphere.2019.124435)

Conclusion

In summary, glyphosate exposure was considered to have caused a block in mouse oocyte development, spindle assembly disruption and chromosome scattered distribution, mitochondrial aggregation and membrane potential reduction, DNA damage, and increased oxidative stress levels, which then led to cellular apoptosis and autophagy. These results were considered to provide evidence for the toxic effects of glyphosate on reproductive systems.

Assessment and conclusion

Assessment and conclusion by applicant:

In vitro intracellular changes in Kunming mice oocytes were evaluated after being cultured in medium supplemented with 500 µM glyphosate. Findings included: decreased germinal vesicle breakdown, decreased first polar body extrusion, increased mRNA expression of anti-oxidant enzyme-related genes, abnormal spindle morphology, increased DNA double strand breaks, aggregated mitochondria, decreased mitochondrial membrane potential, increased protein expression of apoptosis factors, increased mRNA expression of apoptosis related genes and decreased autophagy-related genes.

No dose-response could be determined as only one concentration was tested, far in excess of that considered biologically relevant. Whilst some evaluations were conducted on oocytes harvested from a wider data set of 24 mice (protein expression levels of apoptosis factors by Western blot analysis), a number of the assessments were conducted on oocytes from just 12 mice (mRNA expression of oxidative stress-related, apoptosis-related and autophagy-related genes) or 6 mice (mitochondrial staining, measurement of mitochondrial membrane potential). This narrow source of oocytes limits the robustness of certain conclusions. Furthermore, there are insufficient details reported in the methods to establish whether mice were of the same age before oocyte harvesting or the purity of the glyphosate tested.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterisation of the glyphosate tested, no cytotoxicity testing, the lack of a positive control and insufficient dose-response characterisation at biologically relevant doses.

Reliability criteria made by the applicant

Publication: Zhang JW *et al*., 2019.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	N	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source not reported. Chemical analysis was performed but the results were not clear.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	Not reported	
Test concentrations in physiologically acceptable range (< 1 mM)	Y	50, 100, 200, 500 µM. Most of the assays were carried out at 500 µM which is a concentration that cannot be reached systemically in the rat at 2000 mg/kg bw after oral intake.
Cytotoxicity tests reported	N	
Positive and negative controls	N	No positive control used.
Complete reporting of effects observed	Y	

Publication: Zhang JW *et al*., 2019.	Criteria met? Y/N/?	Comments
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because of the poor characterisation of the glyphosate tested, no cytotoxicity testing and the lack of a positive control.		

Assessment and conclusion by RMS:

In this *in vitro* study, oocytes from Kunming mice were treated with 50, 100, 200 or 500 μM glyphosate to evaluate the ratio of germinal vesicle breakdown (GVBD) and first polar body extrusion (PBE) and with 500 μM glyphosate to evaluate the reactive oxygen species (ROS) levels, spindle morphology, mitochondrial function, DNA integrity, cell apoptosis and autophagy. At least 50 oocytes from 24, 12 or 6 mice were analysed for each experiment, with at least three biological replicates.

At 200 and 500 μM, glyphosate significantly decreased GVBD and PBE indicating adverse effects on oocyte developmental competence. At 500 μM, glyphosate increased the mRNA expression of sod3, gpx and cat genes suggesting enhanced ROS production. There were misaligned chromosomes, abnormal spindle morphology and reduced p-MAPK protein levels in oocytes. The mitochondrial membrane potential was lowered suggesting interference with the mitochondrial function in oocytes. The expression of Bcl-2 protein decreased, while that of Bax protein increased, suggesting induced early apoptosis in oocytes. The mRNA expression of autophagy-related genes (Ic3, atg14 and mtor) and expression of autophagy-related proteins (LC3 and Atg12) was increased suggesting induced autophagy in oocytes.

This findings at the top dose in this in vitro study suggests that glyphosate interfered with mouse oocyte maturation by generation of oxidative stress and early apoptosis. However, no firm conclusion can be made due to several restrictions of the study.

The study is reliable with restrictions as the test material identity, in particular purity, is not specified; only a single concentration level tested in the experiments (except one); and a positive control was missing.

Category A – Johansson, H.K.L. *et al.*

Data point	EU data requirement No. CA 5.6.1/020
Report author	Johansson, H.K.L. *et al.*
Report year	2018
Report title	Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis
Document No.	Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j.reprotox.2018.09.008
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

The aim of the study was to examine the effect of glyphosate and a glyphosate-based herbicide on testis androgen function in adult rats.

Materials and methods

Test substance	Glyphosate (Sigma-Aldrich) and Glyphosate-based herbicide Glyfonova® 450 Plus (FMC Corporation) containing 450 g/L glyphosate acid equivalent.
Lot/Batch	Not specified
Purity/Radiochemical purity	Glyphosate purity ≥ 96 %
Animals	Four–week old male Sprague-Dawley rats were randomly caged in pairs and acclimatised for 7 days. Cages were subsequently distributed into four exposure groups based on animal weight, but following exposure only one animal per cage was used for analyses. Ten animals per dose group were treated orally via gavage with glyphosate at 2.5 (GLY5; 5 times Acceptable Daily Intake) and 25 (GLY50; 50 times ADI) mg/kg bw/day; and with Glyfonova at 25 (NOVA) mg/kg bw/day equivalent glyphosate dose. Water was used as the control. After 2 weeks of exposure, the animals were decapitated under CO_2/O_2 sedation and the testes collected. From each of 10 males per dose group, one testis, selected at random, was snap frozen in liquid nitrogen and the other was fixed in 10 % formalin.
Testosterone assay	One half of each testis was used for hormone extraction. The testes were cut into two equally sized pieces. After removal of the testis cap the inner tissue (seminiferous tubules and interstitial cells) was transferred immediately to a glass vial containing 0.5 mL sterile water and reweighed to determine total tissue weight per sample. Subsequently, 2.5 mL heptane was added and the sample homogenised by manual disruption and then frozen solid. The heptane fraction was transferred and the process repeated to yield a second 2.5 mL heptane fraction that was pooled together with the first fraction. The extracts were dried under nitrogen. Before hormone analyses, the dry extracts were dissolved in EIA buffer at 4 °C overnight, then vortexed and placed in a 42 °C water bath for 10 minutes. Intra-testicular testosterone assays were performed using the Testosterone ELISA kit according to the manufacturer's instructions. Reads were obtained using 96-well plates and absorbance read at 405 nm using a microplate reader. Each sample was assayed in duplicates and result means presented as pg testosterone/g testis.
Histology	Testes were fixed in 10 % formalin, processed for paraffin embedding and sectioned at 5 µm for histological assessments. Hematoxylin & Eosin (H&E) staining was performed following standard protocols.
Immunohistochemistry	Immunohistochemistry with peroxidase was carried out on sections of formalin-fixed testes. Sections were dewaxed in petroleum and rehydrated by immersion in ethanol solutions and finally water. Antigen retrieval was carried out in 0.01 M citrate buffer (pH 6) in a microwave and then allowed to cool at room temperature. Samples were washed in PBS and blocked with 1 % bovine serum albumin (BSA) and then incubated overnight at 4 °C with primary antibody. The following day, samples were washed in PBS, blocked with 3 % H_2O_2 for 10 minutes and again washed in PBS. The samples were incubated with EnVision+System (Dako) for 30 minutes, washed, then with Liquid DAB+System (Dako) for 15 minutes and washed again. To visualize all cell nuclei, sections were counterstained with Meyers' hematoxylin. Following washing and rehydration, samples were mounted with Eukitt. The primary antibody used was against the Androgen receptor.
Immunofluorescence	Five µm sections (4/group) were dewaxed in petroleum and washed in ethanol and dehydrated. Antigen retrieval was done by heat treatment in Tris-EDTA buffer (pH 9) and then cooled at room temp before washing in PBS and blocking in 5 % bovine serum albumin (BSA). The samples were incubated with primary antibodies overnight at 4 °C. The next day, slides were brought to room temperature and incubated with secondary antibodies for 1 hour in the dark. Samples were then counterstained with 4,6-diamidino-2-phenylindole (DAPI). The primary antibodies were goat anti-HSD3β, rabbit anti-

	DDX4, goat anti-CYP11A1 and rabbit anti-STAR. Secondary antibodies were donkey anti-goat AlexaFluor-488 and donkey anti-rabbit AlexaFluor-568.
RNA extraction, cDNA synthesis and quantitative RT-PCR	Total RNA was extracted from a ~100 mg cross-sectional piece from each testis (n=10/group), of which 500 ng RNA was used for each cDNA synthesis. TaqMan Gene Expression Assays were: Ar (Rn00560747), Ddx4 (Rn01489814), Cyp11a1, Cyp17a1, Insl3 (Rn00586632), Pcna (Rn01514538), Star and Hsd3b1 (Rn017747410). RT-qPCR assays were run on a QuantStudio 7 Flex Real-Time PCR System in a 384-well format using 3 µL diluted (1:20) cDNA as template in each 20 µL reaction. Relative transcript abundance was calculated by the comparative Ct-method with the geometric mean of the reference genes Sdha (Rn00590475) and Rpl13a (Rn00821946).
Apoptosis	One section per testis (n=5/group) was stained with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL, Apoptag®Peroxidase in Situ Apoptosis Detection Kit) according to the manufacturer's instructions with one exception i.e. pre-dilution (1:80) of the TdT enzyme in MilliQ water before dilution with reaction buffer. Color was developed for 15 minutes using DAB+substrate. Lastly, the nuclei were stained using Mayer Hematoxylin prior to mounting using Eukitt. All positively stained cells within the seminiferous tubules on a cross-section were counted. Cells that were clearly stained as a result of cell division rather than apoptosis were excluded, as were stained cells at the outer border of the tissue sections. The relative percentage of seminiferous epithelium to whole testis was calculated using a 15 point-grid count on three separate fields of view from each sample. Second, whole testis section areas were calculated in Adobe Photoshop CC 2017 using the measurement tool. Third, apoptotic cells were defined relative to percentage seminiferous epithelium to whole section. Data and statistical analyses are presented as number of apoptotic cells per seminiferous tubuli area.
Statistical analysis	Data were tested for normal distribution and homogeneity of variance and logarithmic transformation was applied if required. ANOVA with Dunnett's post-test was applied and $p<0.05$ was considered statistically significant. Statistical software GraphPad Prism 5 was used for analysis.

Results

Testosterone levels - No statistically significant increase in testicular testosterone levels was observed in rats treated orally with glyphosate at 2.5 and 25 mg/kg bw/day, or with Glyfonova at 25 mg/kg bw/day for 2 weeks (Fig. 1).

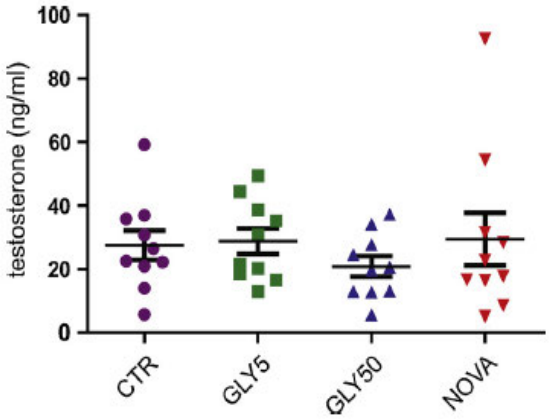

Fig. 1. Intra-testicular testosterone levels were not significantly altered following exposure to glyphosate alone or in a pesticide formulation. Young adult male rats were exposed for two weeks to 2.5 mg/kg bw/day (GLY5), 25 mg/kg bw/day (GLY50) glyphosate, or a 25 mg/kg bw/day equivalent glyphosate dose in a pesticide formulation (NOVA). Intra-testicular testosterone levels were measured from one testis per animal (N = 10 each group) following heptane extraction.

(Figure adapted from Johansson *et al.* (2018). Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis. Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j reprotox.2018.09.008)

Quantitative gene and protein expression analysis - In the testis from animals orally exposed to glyphosate at 2.5 or 25 mg/kg bw/day for 2 weeks no significant differences in gene expression were observed for the Leydig cell specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and the somatic marker gene Ar or germ cell marker gene Ddx4 (Fig. 2).

In the Glyfonova group, there was a small but significant upregulation of Cyp11a1 and Cyp17a1.

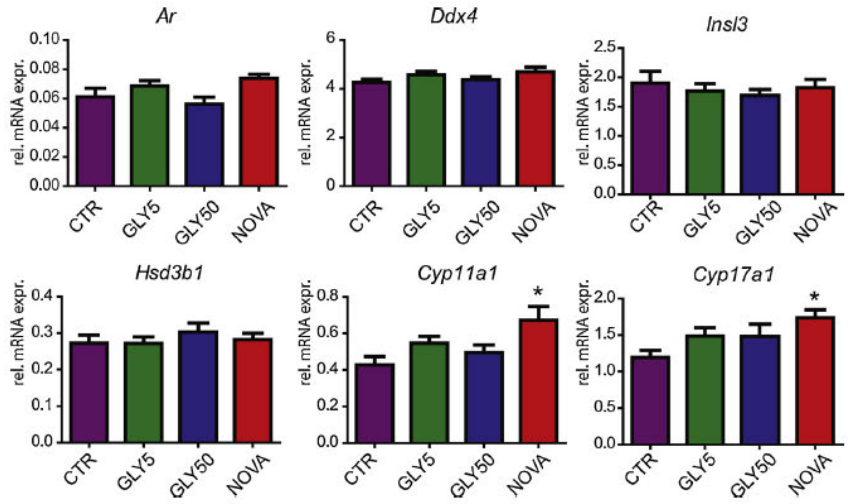

Fig. 2. Expression of genes involved in steroidogenesis was slightly upregulated in testis following exposure to a glyphosate-containing pesticide formulation, but not glyphosate alone. RT-qPCR analysis revealed that expression of *Ar*, the germ cell marker *Ddx4*, and the Leydig cell markers *Insl3* and *Hsd3b1* were unchanged in exposed rats, whereas the Leydig cell-specific steroidogenic genes *Cyp11a1* and *Cyp17a1* were significantly upregulated in rats following two weeks of exposure to 25 mg/kg bw/day glyphosate in a pesticide formulation (NOVA). No significant changes were observed in testis from rats exposed to glyphosate alone. N = 10 each group; *p < 0.05.

(Figure adapted from Johansson *et al.* (2018). Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis. Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j reprotox.2018.09.008)

Testis histopathology - No adverse histopathology in any of the glyphosate or Glyfonova exposed groups was observed when compared with controls. The seminiferous tubules were intact and displayed active spermatogenesis with comparable spermatogenic cycling between examined specimens (qualitative assessment). Neither were there any differences between exposed and control animals with regard to missing germ cell layers or multinucleated germ cells. The interstitial space was comparable between glyphosate treated groups and controls (Fig. 4).

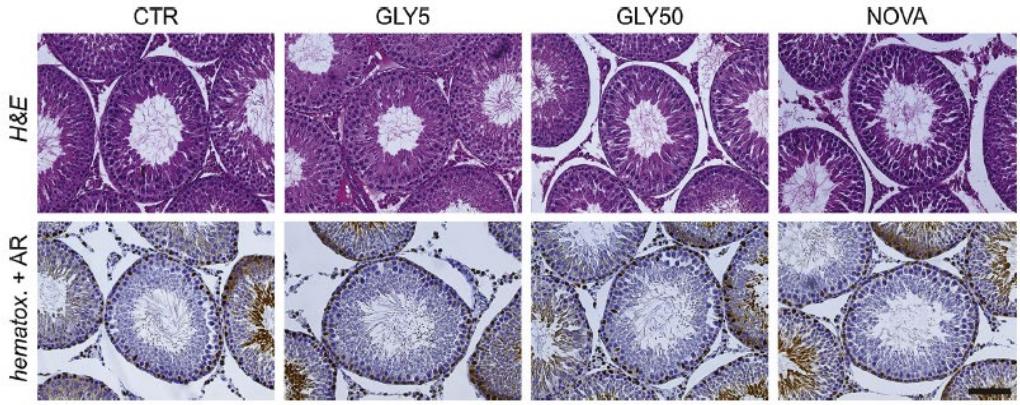

Fig. 4. No histological alterations in testes from glyphosate-exposed rats. Cross-sections of formalin-fixed testes were stained with hematoxylin & eosin (H&E), or immunostained with an antibody against AR (brown) and counterstained with hematoxylin (blue). AR showed strong expression in Leydig, Sertoli, and majority of peritubular cells, with no obvious changes in AR expression or tissue histology in glyphosate-exposed testes. N = 5 each group; scale bar = 100 µm.

(Figure adapted from Johansson et al. (2018). Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis. Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j reprotox.2018.09.008)

Qualitative protein expression analysis - The Leydig cell-specific steroidogenesis factors CYP11A1 and STAR were both expressed at comparative levels between the glyphosate or Glyfonova treated animals and the controls (Fig. 3A). Also no difference in expression and distribution was noted between glyphosate or Glyfonova treated groups and controls for the steroidogenic enzyme HSD3B1 and the germ cell-specific factor DDX4 (Fig. 3B).

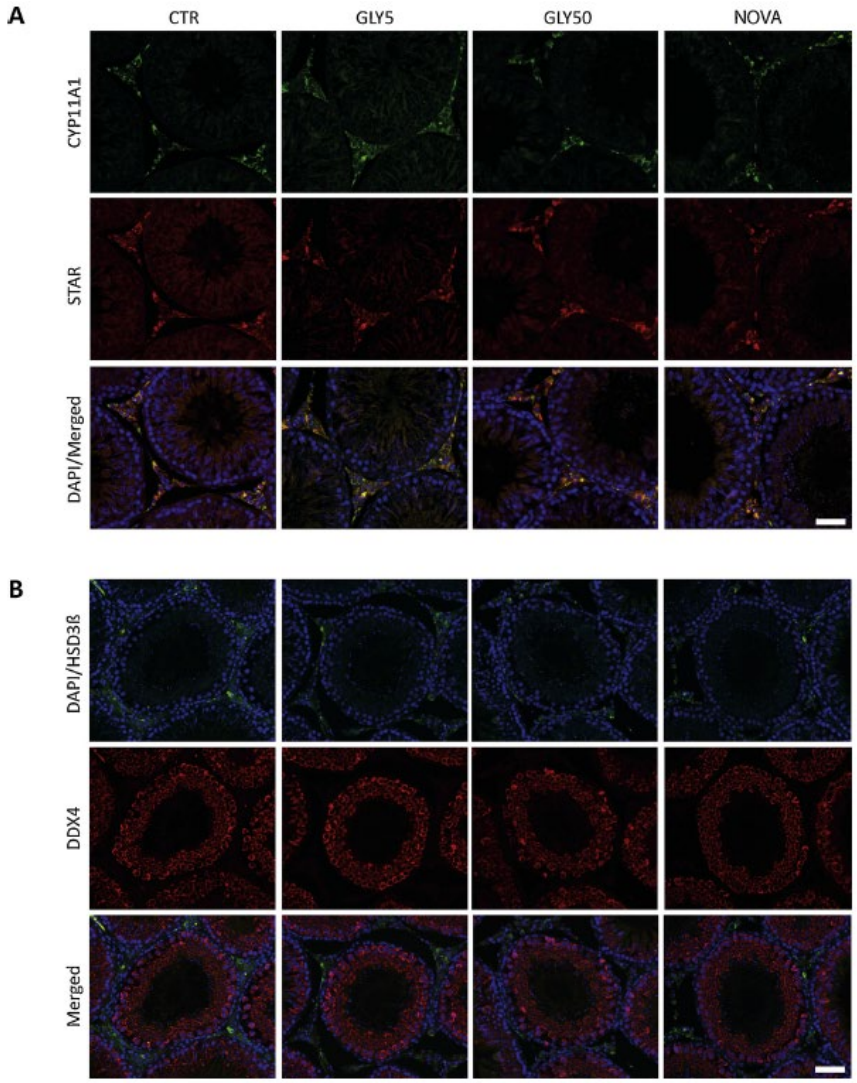

Fig. 3. Assessment of protein expression in testes from glyphosate-exposed rats revealed no obvious alterations. Cross-sections of formalin-fixed testes were immunostained with antibodies against A) two Leydig-cell markers and steroidogenesis factors CYP11A1 (green) and STAR (red), and B) Leydig cell marker HSD3β (green) and germ cell marker DDX4 (red). Samples were counterstained with DAPI (blue). No obvious qualitative changes to expression or tissue histology were observed in any of the samples. N = 5 each group; scale bar = 50 µm.

(Figure adapted from Johansson *et al.* (2018). Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis. Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j reprotox.2018.09.008)

Apoptosis - The number of apoptotic cells was comparable between control testes and glyphosate or Glyfonova treated groups (5 per group) (Fig. 5).

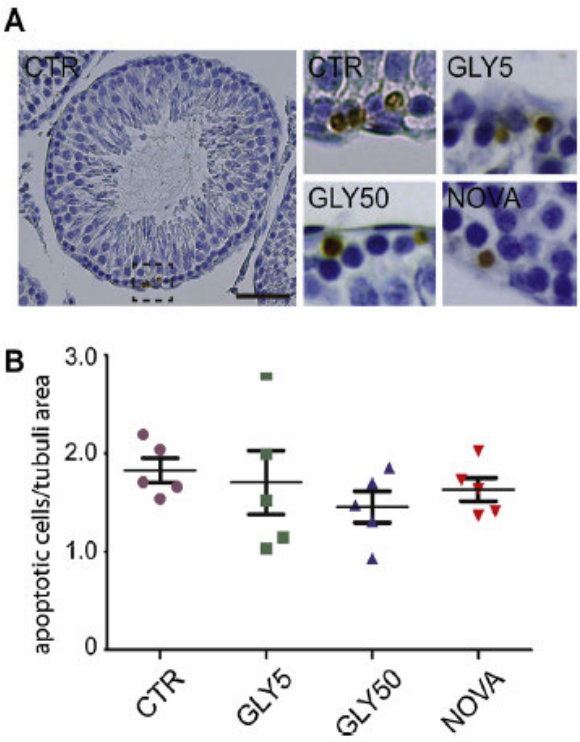

Fig. 5. The rate of apoptosis was comparative between glyphosate-exposed and control testes. A) Cross-section of formalin-fixed testes were stained with TUNEL (brown) and counterstained with hematoxylin (blue). TUNEL-positive cells within the seminiferous tubules that were not obviously in cell division were counted across the entire section. B) The number of apoptotic cells were comparable between control testes and all exposure groups. N = 5 each group; scale bar = 50 µm.

(Figure adapted from Johansson *et al.* (2018). Exposure to a glyphosate-based herbicide formulation, but not glyphosate alone, has only minor effects on adult rat testis. Reproductive Toxicology, Vol. 82, December 2018, Pages 25-31 doi.org/10.1016/j reprotox.2018.09.008)

Assessment and conclusion

Assessment and conclusion by applicant:

The effects of glyphosate on intra-testicular testosterone levels, expression of Leydig cell-specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3β1 and Star and expression of somatic marker gene Ar or germ cell marker gene Ddx4, expression of Leydig cell-specific steroidogenesis factors CYP11A1 and STAR, testicular histopathology and apoptosis were investigated in male rats treated orally at 0, 2.5 and 25 mg/kg bw/day for 2 weeks. No effects were found on either of the testicular parameters tested suggesting that glyphosate does not contribute to endocrine disrupting effects of the male reproductive system.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.

Reliability criteria made by the applicant

Publication: Johansson et al., 2018.	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following	Y	

scientifically acceptable standards		
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity ≥ 96 %. Source: Sigma-Aldrich, St Louis, USA.
Only glyphosate acid or one of its salts is the tested substance		Also formulations were tested: Glyfonova 450 Plus, FMC corporation.
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral by gavage.
Dose levels reported	Y	Only 2 dose levels for glyphosate (2.5 and 25 mg/kg bw)
Number of animals used per dose level reported	Y	10 animals/group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Only 2 dose levels tested.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only two dose levels were used to explore the dose-effect relationship for the endpoints assessed.		

Assessment and conclusion by RMS:

In this study, adult Sprague-Dawley male rats (10/group) were treated via gavage with glyphosate at 2.5 and 25 mg/kg bw/day; and with a glyphosate-based herbicide Glyfonova at 25 mg/kg bw/day equivalent glyphosate dose for two weeks. Following parameters were investigated: intra-testicular testosterone levels; expression of key marker genes in the testes; testis histopathology, protein expression analysis and apoptotic activity.

Glyphosate or Glyfonova had no significant effect on testosterone levels. There were no effects of glyphosate treatment on expression of Leydig cell-specific genes Cyp11a1, Cyp17a1, Insl3, Hsd3b1 and Star, the somatic marker Ar or germ cell marker Ddx4. However, Glyfonova treatment led to statistically significant upregulation of Cyp11a1 and Cyp17a1. There were no effects of glyphosate or Glyfonova on: the relative expression levels and distribution of the steroidogenic enzyme HSD3B1 and the germ cell-specific factor DDX4; testis histopathology; or apoptotic rate.

Overall, in this study, glyphosate had no effect on testis parameters investigated while Glyfonova showed effects on steroidogenic gene expression.

The study is relevant for risk assessment but reliable with restrictions as the exposure duration was short (2 weeks only), endpoints were limited (for e.g., testes were not weighed) and there were only 2 dose levels (for glyphosate; and only 1 for Glyfonova).

Category A – Panzacchi, S. *et al.*

Data point	CA 5.6.1/021
Report author	Panzacchi, S. *et al.*
Report year	2018
Report title	The Ramazzini Institute 13–week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation
Document No.	doi.org/10.1186/s12940-018-0393-y ISSN: 1476-069X
Guidelines followed in study	Design of the study derives from the 13-week cohort protocol of the National Toxicology Program's (NTP) Modified One-Generation Reproduction Study 2011 (as stated in article)
Deviations from current test guideline	No deviations specified in article
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	Yes
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

This pilot study represents the first phase of an integrated long-term project on glyphosate-based herbicides. The initial focus of the pilot study is to assess techniques and methods for glyphosate detection in different matrices, then to evaluate target organ toxicity, genotoxicity and endocrine disrupting activities, together with omics and microbiome alterations. In this paper, data on general toxicity and urinary concentrations of glyphosate and its major metabolite AMPA was presented.

Materials and methods

Test substance	Glyphosate (Sigma-Aldrich) and Glyphosate-based herbicide Roundup Bioflow (MON 52276) (Consorzio Agrario dell´ Emilia, Bologna, Italy) containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5 %), water (42.5 %) and surfactant (16 %)
Lot/Batch	Not specified
Purity/Radiochemical purity	Glyphosate purity ≥ 99.5 %
Animals	Male and female SD rats were obtained from the colony used at the Cesare Maltoni Cancer Research Center laboratories of the Ramazzini Institute (CMCRC/RI). The animal room conditions were 22 ± 3 °C and 50 ± 20% relative humidity and a light/dark cycle of 12 hours. During the experiment the animals received standard pellet feed and tap water *ad libitum.*
Experimental design	Each of 8 virgin female SD rats (17 weeks old, 270-315 g) per dose group was placed individually in a polycarbonate cage with a single male rat of the same age and strain until evidence of copulation. Gestational day (GD) 0 was defined as the day on which sperm was found in vaginal smears. After mating, matched females were housed separately during gestation and delivery and pups were housed with the dams until weaning. The day on which parturition was completed was designated as lactating day (LD) 0 for the dam and postnatal day (PND) 0 for the offspring. On PND 28, the offspring were weaned and identified by ear punch. Sequentially, they were allocated in the same treatment group of their mother to obtain 18 animals (8 for the 6–week cohort and 10 for the 13–week cohort) per sex and for each dose group. No more than 2 males and 2 females from the same litter were included in the same cohort/treatment group. Altogether, 54 males and 54 females were enrolled in the post-weaning treatment phase. Rats were treated with glyphosate and MON 52276 at 1.75 mg glyphosate acid eq./kg bw/day in the drinking water. One group received only tap water as control. After weaning, until the end of the experiment (PND 73 ± 2 or 125 ± 2), glyphosate and MON 52276 were administered in the drinking water to F1 animals on the basis of the average body weight and average water consumption per sex and per experimental group. Males and females were considered separately because of their difference in weight gain, body

	weight and water consumption. Animals were checked 3 times daily on working days and 2 times daily on Sundays and non-working days. Clinical signs were checked before the start of the treatment, and at least every two days until the end of the experiment. The body weight of the dams was recorded on GD 0, 3, 6 and then daily during gestation until parturition. During lactation, the body weight of the dams was recorded at LD 1, 4, 7, 10, 13, 16, 19, 21 and 25 and the body weight of the pups by sex and litter was determined on PND 1, 4, 7, 10, 13, 16, 19, 21 and 25. After weaning, body weight was measured twice a week until PND 73 ± 2, then weekly until PND 125 ± 2 and before terminal sacrifice. The mean individual body weights were calculated for each group and sex. Feed and water consumption of the dams were recorded twice weekly on GD 0, 3, 6, 9, 12, 15, 18, 21, and during lactation on LD 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28. After weaning the daily feed and water consumption per cage were measured twice a week, until PND 73 ± 2, then weekly until PND 125 ± 2. The mean individual feed and water consumption were calculated for each group and sex. The day before terminal sacrifice, all the animals were placed individually in metabolic cages and starved for around 16 hours during which the animals had free access to water alone or to the test compound solutions. In the morning of the following day, samples of at least 5 mL of urine collected from each animal were transferred to labelled tubes for analysis of glyphosate and AMPA. Samples from 3 dams/group and from 5 rats/sex/group belonging to the 6–week and 13–week cohorts were used for analysis. Summary of the endpoints and relative monitoring time points in the study, in dams and offspring is presented in Table 2.
Analysis of glyphosate and AMPA in urine	Analysis of glyphosate and AMPA in drinking water, feed and urine were performed by Neotron Laboratories. The specification and results are maintained in the experimental documentation. Analysis was performed using LC-MS/MS. The limit of quantification (LOQ) for glyphosate and AMPA was 0.10 µg/L in water, 50 µg/kg in feed, and 1 µg/L in urine.
Statistical analysis	Means ± standard deviations (SD), were calculated for continuous variables. For body weight, water and feed consumption over time multilevel mixed-effect linear regression models were used, to control for within subject correlation across time also considering the litter effect during the lactation period. Analysis of variance and Dunnett's test (when applicable) were also performed to compare body weight gain in different periods and consumption of food and water as mean consumption in several periods. All tests were two tailed with results considered as statistically significant if $p<0.05$. Statistical analyses were performed by using STATA version10.

Table 1 Experimental plan

Breeders			Offspring							
Group	Animals		Group	Animals[a]			Treatment[b]			End of the experiment
	Sex	No.	N.	Sex	Cohort		Compound	Dose[c]	Age at start[d]	Cohort
					6-week (No.)	13-week (No.)				6-week (PND) / 13-week (PND)
I	F	8	I	M	8	10	Control (drinking water)	0	GD 6	70[e] / 120[f]
	M	8		F	8	10				
	F + M	16		M + F	16	20				
II	F	8	II	M	8	10	Glyphosate	US ADI	GD 6	70[e] / 120[f]
	M	8		F	8	10				
	F + M	16		M + F	16	20				
III	F	8	III	F	8	10	Roundup	US ADI Glyphosate equivalent	GD 6	70[e] / 120[f]
	M	8		M	8	10				
	F + M	16		F + M	16	20				
Total	M + F	48		M + F	48	60				

[a] No more than 2 sisters and 2 brothers per litter
[b] Test compounds are administered ad libitum in drinking water
[c] Doses are calculated considering the Glyphosate US ADI defined as the chronic Reference Dose (cRfD) determined by the US EPA (1.75 mg/kg bw/day)
[d] Solutions are administered to dams starting from the 6th day of pregnancy
[e] Animals are treated until the landmarks of sexual development are acquired (PND 73 ± 2)
[f] Animals are treated from embryonic life (GD 6) indirectly from dams milk until PND 28 ± 2, then directly for 90 days after weaning (until PND 125 ± 2)

(Table adapted from Panzacchi *et al.* (2018). The Ramazzini Institute 13–week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. Environmental Health17:52 doi.org/10.1186/s12940-018-0393-y)

Table 2 Summary of the endpoints and relative monitoring time points evaluated in the study, in dams and offspring (6-week and 13-week cohorts)

Endpoints	Time points	Dams	Offspring 6-week cohort	Offspring 13-week cohort
Gestation length	GD0-delivery	✓	–	–
AGD and body weight in male and female pups	PND 1	–	✓	✓
Litter size	PND 1, 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Live-birth index	PND 1	–	✓	✓
Survival index	PND 4, 7, 10, 13, 16, 19, 21, 25	–	✓	✓
Age and body weight at BPS in male pups	PND 35	–	✓	✓
Age and body weight at VO in female pups	PND 28	–	✓	✓
First estrous in female pups	3 days after VO	–	✓	–
Estrous cycle length and percentage of days in each stage	PND 95 - PND 116	–	–	✓
Estrous cycle prior to necropsy	PND 125 ± 2	–	–	✓
Serum hormone measures	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Clinical biochemistry	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Urinalysis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Glyphosate and AMPA detection in urine	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Sperm counts	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Daily Sperm production	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm transit time through the epididymis	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm morphology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Sperm aneuploidy	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Partial histopathology (reproductive organs, brain, liver, kidney)	End of lactation (dams)	✓	–	–
Complete histopathology	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Organ weight	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Micronuclei test (bone marrow)	PND 73 ± 2 and PND 125 ± 2	–	✓	✓
Transcriptome on mammary glands	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on brain	PND 125 ± 2	–	–	✓
Transcriptome on liver	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Transcriptome on kidneys	End of lactation (dams), PND 73 ± 2 and PND 125 ± 2	✓	✓	✓
Microbiome analysis in dams	Before mating, GD 5 (before treatment), GD 13, LD 7, LD 14	✓	–	–
Microbiome analysis in offspring	PND 7, PND 14, PND 31 (before puberty), PND 57 (after puberty), PND 125 ± 2 (adulthood)	–	✓	✓

GD gestation day, *LD* lactation day, *PND* postnatal day, *AGD* anogenital distance, *VO* vaginal opening, *BPS* balano preputial separation

(Table adapted from Panzacchi *et al.* (2018). The Ramazzini Institute 13–week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. Environmental Health 17:52 doi.org/10.1186/s12940-018-0393-y)

Results

Mortality, body weight, water and food consumption, clinical signs and litter data - All 24 dams and 108 rats from the 6–week and 13–week cohorts survived until sacrifice. Body weight and body weight gain of the dams during gestation and lactation were not statistically different among groups. Post-weaning body weight of female and male offspring was homogeneous and no statistically significant differences in body weight gain were observed among groups. Water and feed consumption during gestation and lactation were not different across groups. Litter sizes were fully comparable among groups, with mean number of live pups of 13.6 (range 10–16) in the control group, 13.3 (range 11–17) in the glyphosate group and 13.9 (range 11–16) in the MON 52276 group. Post weaning water and feed consumption were not affected by treatment. There was no clinical evidence of alterations in activity or behavior, reflexes, eyes or skin, respiratory, gastrointestinal, genitourinary and cardiovascular systems.

Analysis of glyphosate and AMPA in urine – The results of the analysis are presented in the Table below.

Table. Glyphosate and AMPA concentration in urine. Results are reported as mean ± standard deviations

		Dams		Offspring (6-week cohort)		Offspring (13-week cohort)	
	Treatment	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)	Glyphosate (mg/kg)	AMPA (mg/kg)
Male	Control			0.012 ± 0.010	0.003 ± 0.003	0.011 ± 0.010	0.006 ± 0.004
	Glyphosate	_	_	0.938 ± 0.414	0.014 ± 0.007	1.684 ± 0.768	0.023 ± 0.012
	Roundup			1.174 ± 0.439	0.011 ± 0.005	2.280 ± 1.520	0.027 ± 0.016
Female	Control	0.009 ± 0.001	0.006 ± 0.002	0.013 ± 0.007	0.005 ± 0.001	0.008 ± 0.005	0.003 ± 0.005
	Glyphosate	0.480 ± 0.010	0.024 ± 0.002	0.938 ± 0.377	0.016 ± 0.010	1.354 ± 0.359	0.013 ± 0.006
	Roundup	0.700 ± 0.106	0.024 ± 0.001	0.910 ± 0.383	0.018 ± 0.007	1.524 ± 0.585	0.021 ± 0.007

(Table adapted from Panzacchi *et al.* (2018). The Ramazzini Institute 13–week study on glyphosate-based herbicides at human equivalent dose in Sprague Dawley rats: study design and first in-life endpoints evaluation. Environmental Health (2018) 17:52 doi.org/10.1186/s12940-018-0393-y)

The urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON52276 at 1.75 mg glyphosate acid eq. /kg bw/day, despite the limited sample size and the large standard deviations. Glyphosate and AMPA urinary levels were all below or close to the LOQ of 0.001 mg/kg in the control group. In the treated rats, the majority of glyphosate was excreted unchanged, with urinary levels about 100-fold higher than that of AMPA. For example, the mean urinary levels of glyphosate were 1.354 and 1.524 mg/kg bw in glyphosate and MON 52276 treated females in the 13–week cohort, respectively, while the corresponding AMPA levels were 0.013 and 0.021 mg/kg bw. In glyphosate and MON 52276 treated rats, a time-dependent increase in the mean urinary concentration of glyphosate was observed. In glyphosate and MON 52276 treated males, an increase of approximately 2-fold was observed of the mean urinary concentration of glyphosate in the 13–week cohort when compared to the 6–week cohort. In glyphosate treated females, the 6–week cohort showed a 2-fold higher mean urinary concentration of glyphosate than the dams after weaning, while the 13–week cohort showed a 1.5-fold increase compared to the 6–week cohort. In the MON 52276 group, the increase was less steep, but the time-dependent pattern was still evident. In glyphosate and MON 52276 treated rats, the levels of AMPA were comparable at the different time points in both males and females. Large variations were observed of the AMPA concentrations in urine, in particular those close to the LOQ as in the control groups.

Discussion

Survival, body weights, food and water consumption of rats were not affected by the treatment with glyphosate or MON 52276. No clinical changes were observed in the animals of the dosed groups. Overall, oral treatment of glyphosate and MON 52276 via the drinking water seemed to be well tolerated. Exposure to glyphosate and MON 52276 led to comparable concentrations of glyphosate and AMPA in urine, indicating that systemic exposure does occur at the selected exposure level of 1.75 mg/kg bw/day, corresponding to the US ADI. The bioavailability of glyphosate in this study is also supported by the evident increase of glyphosate concentration in urine in relation to the length of treatment. The adjuvants and the other substances present in MON 52276 did not seem to exert a major effect on the absorption and excretion of glyphosate, even though mean values of glyphosate seem to be somewhat higher in the formulation treated group. The levels in urine were also comparable between the two sexes, although a consistent inter-individual variability was observed. In rats, glyphosate in urine appears to be the most accurate biomarker of exposure to glyphosate based herbicides (GBHs). The results from this study confirm previous evidence that in rodents most of the administered dose of glyphosate (98 %) is excreted as unchanged parent compound, whereas the metabolite AMPA in urine is at around 0.2–0.3 % of the administered dose. Furthermore, with the level of exposure to glyphosate used in this pilot study, AMPA urinary values of treated animals (0.011–0.027 mg/kg) were already close to the LOQ (0.001 mg/kg) which might limit the reliability of the data. Glyphosate concentrations in urine of treated animals (0.480–2.280 mg/kg) were found to be 100-fold higher than the AMPA concentrations and at least 500-fold higher than the LOQ. Therefore, in order to assess exposure to glyphosate in rats, in particular at doses that are equal or lower than 1.75 mg/kg bw/day, glyphosate appears to be the biomarker of choice. The presence of negligible levels of glyphosate (0.003–0.013 mg/kg) in some of the urine samples of the control groups might reflect an ubiquitous environmental contamination at ultra-low doses of glyphosate, which is consistent with previous reports from other authors. As the current LOQ of glyphosate in HPLC for pelleted animal feed is 0.050 mg/kg, this represents a technical limiting factor for the testing ultra-low doses of glyphosate.

Conclusion
A pilot study was performed on the health effects of glyphosate and its formulation Roundup Bioflow (MON 52276) administered orally to rats at the US ADI of 1.75 mg/kg bw/day. Treatment with either glyphosate or MON 52276 seemed to be overall well tolerated, consistent with previous experiments performed by the US NTP. Both glyphosate and MON 52276 exposure led to comparable urinary concentrations of glyphosate and AMPA with an increasing excretion of glyphosate in urine with duration of treatment. This indicates the systemic bioavailability of glyphosate and a possible mechanism of bioaccumulaton. The adjuvants and the other substances present in the formulation did not seem to exert a major effect on the absorption and excretion of glyphosate when administered orally. The results of this study confirm that, in rodents, glyphosate is a much more relevant biomarker in urine than AMPA, in particular at doses that are equal or lower than 1.75 mg/kg bw/day.

Assessment and conclusion

Assessment and conclusion by applicant:
In this study the general toxicity of glyphosate was compared against that of its reference formulation MON 52276 in pregnant rats and their progeny. Also, the urinary excretion of glyphosate and AMPA was investigated. The test compounds were administered via the drinking water resulting in a daily dose of 1.75 mg glyphosate acid eq./ kg bw. The endpoints investigated were mortality, body weight, water and food consumption, and clinical signs in dams and offspring and litter data. There was no mortality and no statistically significant differences were observed among control, glyphosate, and MON 52276 groups in any of the endpoints investigated. Urinary concentrations of glyphosate and AMPA of rats treated with glyphosate at 1.75 mg/kg bw/day were comparable to those observed in rats treated with MON 52276 at 1.75 mg glyphosate acid eq. /kg bw/day. This indicates that the co-formulants in this glyphosate formulation have little influence on the oral bioavailability of glyphosate. In the treated rats, the majority of glyphosate was excreted in urine unchanged at levels of about 100-fold higher than that of AMPA and the mean urinary concentration of glyphosate increased with the duration of treatment.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.

Reliability criteria made by the applicant

Publication: Panzacchi *et al*., 2018	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	Only a part of the reproductive toxicology study was reported, one dose level of glyphosate or MON 52276 was considered and 8 females per dose group were used.
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	For the part that was reported.
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Glyphosate (purity of > 99,5 %), Pestanal™ analytical standard purchased from Sigma-Aldrich (Milan, Italy).
Only glyphosate acid or one of its salts is the tested substance	N	The representative formulated product, Roundup Bioflow (MON 52276, containing 360 g/L of glyphosate acid in the form of 480 g/L isopropylamine salt of glyphosate (41.5 %), water (42.5 %) and surfactant (16 %) was purchased from Consorzio Agrario

		dell'Emilia, Bologna, Italy.
AMPA is the tested substance	Y	Determined in urine of rats treated with glyphosate and MON 52276.
Study		
Test species clearly and completely described	Y	Male and female SD rats.
Test conditions clearly and completely described	Y	
Route and mode of administration described	Y	Oral via drinking water.
Dose levels reported	Y	1.75 mg glyphosate acid eq./kg bw/day administered as glyphosate and as MON 52276.
Number of animals used per dose level reported	Y	8 virgin female SD rats per dose group.
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Method of analysis described for analysis of urine	N	No details on the conduct of the method of analysis and no complete validation data set.
Complete reporting of effects observed	Y	Limited to body weight,
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	N	Not possible with one dose level of each test item.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because only one dose level for glyphosate and MON 52276 was considered, only 8 animals were used per dose and per sex and the method of analysis of glyphosate and AMPA in urine and its validation were not fully reported.		

Assessment and conclusion by RMS:

In this study, Sprague-Dawley rats were orally via drinking water exposed to 1.75 mg/kg bw/day starting from prenatal life, i.e. gestational day (GD) 6 of their mothers. One cohort was continuously dosed until sexual maturity (6-week cohort) and another cohort was continuously dosed until adulthood (13-week cohort). The endpoints investigated were mortality, body weight, water and food consumption, and clinical signs in dams and offspring and litter data. Survival, body weights, food and water consumption of rats were not affected by the treatment with glyphosate or MON 52276. No clinical changes were observed in the animals of the dosed groups. Furthermore, litter sizes were fully comparable among groups. In the treated rats, the majority of glyphosate was excreted in urine unchanged at levels of about 100-fold higher than that of AMPA and the mean urinary concentration of glyphosate increased with the duration of treatment. The study is limited (low dose only, few animals, actual levels of test compounds that reached the foetus during gestation or that were ingested postnatally by the offspring during the period of lactation were not estimated, method of analysis of glyphosate and AMPA in urine and its validation were not fully reported).

Category A – Perego, M.C. *et al.*

Data point	CA 5.6.1/022
Report author	Perego, M.C. *et al.*
Report year	2017
Report title	Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*
Document No.	DOI 10.1002/jat.3417 E-ISSN: 1099-1263
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	None
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restriction

Aim of the study

The aim of this study was to determine the potential endocrine disruptor effects of glyphosate on ovarian function evaluating cell proliferation, steroidogenesis and gene expression using bovine granulosa cells (GC) and theca cells as *in vitro* models.

Materials and methods

Test substance	Glyphosate (Sigma-Aldrich)
Lot/Batch	Not specified
Purity/Radiochemical purity	Not specified
Cell culture	Ovaries from non-pregnant beef heifers were collected from a local slaughterhouse and were treated as previously described (Lagaly *et al.,* 2008; Langhout *et al*., 1991; Spicer & Aad, 2007). Thecal cells (TC) were collected from large (8–22 mm) follicles as previously described (Lagaly *et al.,* 2008; Spicer & Chamberlain, 1998; Stewart *et al.,* 1995). Follicular fluid was collected and final cell preparations were prepared in serum-free medium (Dulbecco's modified Eagle medium and Ham's F12) as previously described (Lagaly *et al.,* 2008; Schreiber & Spicer, 2012). Trypan blue exclusion method was performed to determine viable cells (Langhout *et al*., 1991; Spicer *et al.,* 1993; Tiemann *et al*., 2003 a,b). Cells were then plated (2.5×10^5 in 20–80 µL of medium) on 24-well Falcon multiwell plates (Becton Dickinson, Lincoln Park, NJ, USA) in 1 mL of basal medium composed of a mixture of 1:1 Dulbecco's modified Eagle medium and Ham's F-12 containing glutamine, gentamicin and sodium bicarbonate (Sigma-Aldrich Co., St. Louis, MO, USA) as previously described (Schreiber & Spicer, 2012). Plates were maintained in a humidified 95% air and 5% CO_2 environment at 38.5 °C changing medium every 24 h. Cells were also kept in the presence of 10% foetal calf serum (FCS; Equitech-Bio, Inc., Kerrville, TX, USA) for the first 48 h of culture to procure an optimal attachment. After 48 h, cells were washed twice with serum-free medium and the different treatments were applied in serum-free medium containing 500 ng/mL of testosterone (as an estradiol [E2] precursor; from Steraloids, Wilton, NH, USA) for 48 h. Ovine follicle-stimulating hormone (FSH; NIDDK-oFSH-20, activity: 175 × NIH-FSH-S1 U mg-1, from the National Hormone and Pituitary Program, Torrance, CA, USA) was added to all treatments, because insulin-like growth factor (IGF) 1 alone does not have an effect on steroid production (Ranzenigo *et al*., 2008; Spicer *et al*., 2002). After aspiration of follicular fluid, large follicles were bisected and GC were separated from the TC via blunt dissection and the theca interna was enzymatically digested as previously described (Aad *et al.,* 2006; Spicer & Chamberlain, 1998; Stewart *et al*., 1995). The non-digested tissue was eliminated by using sterile syringe filter holders with metal screens of 149 µm mesh (Gelman, Ann Arbor, MI, USA) and filtered TC were then

centrifuged at 50 g for 5 min. As described for granulosa cells (GC), TC were washed with serum-free medium and resuspended in serum-free medium containing collagenase and DNase. TC (2.0×10^5 viable cells per well) were plated and cultured as described for GC. Culture medium was also supplemented with 30 ng/mL of ovine luteinizing hormone (LH; LH activity; 2.3 × NIH-LH-S1 U mg^{-1}; from the National Hormone and Pituitary Program) because progesterone (P4) and androstenedione (A4) production are not induced by IGF1 in the absence of LH (Spicer & Stewart, 1996; Stewart *et al.,* 1995).

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Assays	Medium collected from individual wells was frozen at -20 °C for subsequent steroid analyses. Radioimmunoassays (RIA) were performed to determine concentrations of P4, E2 and A4 as previously described (Lagaly *et al.,* 2008; Langhout *et al.,* 1991; Spicer *et al*., 1993; Stewart *et al.,* 1995). The intra- and inter-assay coefficients of variation were 7 % and 13 % for the P4 RIA, and 8 % and 17 % for the E2 RIA, respectively.
Determination of granulosa cell and theca cell numbers	The numbers of GC and TC, in the same wells from which medium was collected, were determined via a Coulter counter (model Z2; Beckman Coulter, Inc., Hialeah, FL, USA), and used to calculate steroid production on ng or pg per 105 cell basis. Cells were washed twice using 0.9 % saline solution (500 µL), exposed to 500 µL of trypsin (0.25 % wt/vol= 2.5 mg/mL) for 20 min at 37 °C and then scraped from each well and enumerated as previously described (Lagaly *et al*., 2008; Ranzenigo *et al.,* 2008).
RNA extraction and quantitative reverse transcription-polymerase chain reaction	At the end of the treatment period, medium was either aspirated or collected from each well depending on the experiment and cells from two replicate wells were lysed in 0.5 mL of TRI reagent solution (Life Technologies, Inc., Grand Island, NY) as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007). RNA samples were solubilised in DEPC-treated water (Life Technologies, Inc., Grand Island, NY), quantitated by spectrophotometry at 260 nm using a NanoDrop ND-1000 spectrophotometer, and stored at -80 °C. Cholesterol side chain cleavage enzyme (CYP11A1) and aromatase (CYP19A1) primers and probes for quantitative reverse transcription–polymerase chain reaction were designed using Primer Express™ software (Foster City, CA, USA) as previously reported (Lagaly *et al.,* 2008; Spicer & Aad, 2007). The bovine CYP11A1 and CYP19A1 primer and probe sequences and information are described by Lagaly *et al.* (2008). Relative quantification of target gene mRNAs were expressed using the comparative threshold cycle method as previously described (Lagaly *et al*., 2008; Spicer & Aad, 2007).
Experimental design	Experiment 1 was performed to evaluate the effects of glyphosate (GLY) on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10% FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL; recombinant human IGF1 from R&D Systems, Minneapolis, MN, USA) with or without various doses of GLY (i.e., 0, 0.5, 5 µg/mL; Sigma-Aldrich Co.). Only the 0 and 5 µg/mL doses of GLY were tested in the absence of IGF1 to determine any possible effect of GLY on FSH-stimulated steroidogenesis. After 48 h of treatment, cells were counted and medium was collected for E2 and P4 determinations. In a separate set of cells, the effect of 2–day treatment with 5 µg/mL GLY on GC viability was evaluated using the trypan blue exclusion method as previously described (Adashi *et al.,* 1987; Spicer & Alpizar, 1994*). *Spicer LJ, Alpizar E. 1994. Effects of cytokines on FSH-induced estradiol production by bovine granulosa cells in vitro: dependence on size of follicle. Domest. Anim. Endocrinol. 11: 25–34. Experiment 2 was designed to evaluate the effects of higher doses of GLY on GC proliferation and steroidogenesis. Cells were cultured for 48 h in 10 % FCS, washed twice with serum-free medium as described earlier, and cells treated for 48 h in serum-free medium containing testosterone (500 ng/mL), FSH (30 ng/mL) and IGF1 (30 ng/mL) with or without the various doses of GLY (i.e., 0, 0.01, 0.3 mg mL-1). After 48 h of treatment, cells were counted and medium was collected for E2 and P4 determinations. Experiment 3 was designed to test the effect of GLY on CYP11A1 and CYP19A1 mRNA abundance in GC. Cells were cultured as previously described for experiment 1 except that no testosterone was included in the medium and treatments were only applied for 24 h: no additions, FSH (30 ng/mL) plus IGF1 (30 ng/ mL), and FSH plus IGF1 plus GLY (5 µ/mL). After 24 h of treatment, cells were lysed for RNA extraction as described earlier. A combined treatment of FSH and IGF1 was selected to test the GLY effect because the inhibitory effect of GLY was seen with this treatment in experiment 1. Experiment 4 was performed to study the effect of GLY on TC proliferation and steroidogenesis. Cells were cultured for 48 h in 10 % FCS, washed twice with serum-free

	medium as described earlier, and cells treated for 48 h in serum-free medium containing LH (30 ng/mL) and IGF1 (0 or 30 ng/mL) with or without GLY (i.e., 0 and 5 µg/mL). After 48 h of treatment, cells were counted and medium was collected for P4 and A4 determinations. Experiment 5 was designed to determine the effect of GLY on serum-stimulated GC proliferation. Cells were cultured for 4 days in 10 % FCS. During the last 2 days of culture, cells were treated as follows: control (no additions) or GLY (1.7 µg/mL). At the end of treatment, cells were counted.
Statistical analysis	Experimental data are presented as the least squares means ± SEM of measurements from replicated culture wells. Each experiment was performed three times with different pools of GC collected from 10 to 20 ovaries for each pool and each treatment replicated three times within each experiment. Treatment effects and interactions were assessed using the GLM procedure of the Statistical Analysis System for Windows (version 9.2; SAS Inst. Inc., Cary, NY, USA). Main effects were treatment, experimental replicate (i.e., pool of cells) and their interaction. Steroid production was expressed as ng or pg per 10^5 cells per 24 h, and cell numbers determined at the end of the experiment were used for this calculation. Mean differences in cell growth and steroid production between treatments were determined using the Fisher's protected least significant difference procedure (Ott, 1977) $P<0.05$ was considered statistically significant.

Results

Experiment 1: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone with or without insulin-like growth factor 1

GLY at 0.5 and 5 µg/mL was found to decrease significantly the GC proliferation in the presence of FSH plus IGF1 (Fig. 1). Regarding steroid production, GLY at all tested concentrations (0.5 and 5 µg/mL) had no effect on GC P4 production (Fig. 2A). GLY had no effect on GC E2 production in the presence of FSH whereas GLY at 5 µg/mL decreased ($P<0.05$) E2 production in the presence of FSH plus IGF1 (Fig. 2B). Cell viability was not significantly affected by 2–day treatment with 5.0 µg/mL GLY (91.6 vs. 88.6±5.0 % for control and GLY-treated GC, respectively).

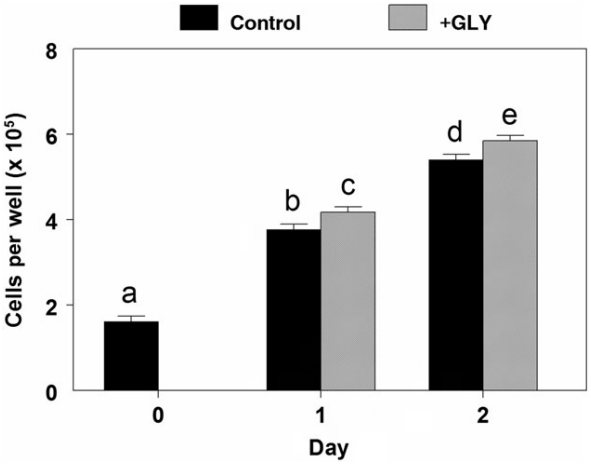


Figure 1. Effect of GLY on numbers of granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng/mL FSH alone and GLY at 0 or 5 µg/ mL, or with 30ng/mL FSH and IGF1 (30 ng/mL) with GLY at 0, 0.5 or 5.0 µg/mL. All cells were treated concomitantly with 500 ng/mL of testosterone. Values are means ± SEM from three separate experiments (n = 9). Means without a common letter (a d) differ ($P<0.05$). FSH, follicle-stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.
(Figure adapted from Perego *et al.* (2017). Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*. Journal of Applied Toxicology 37:692-698. DOI 10.1002/jat.3417)

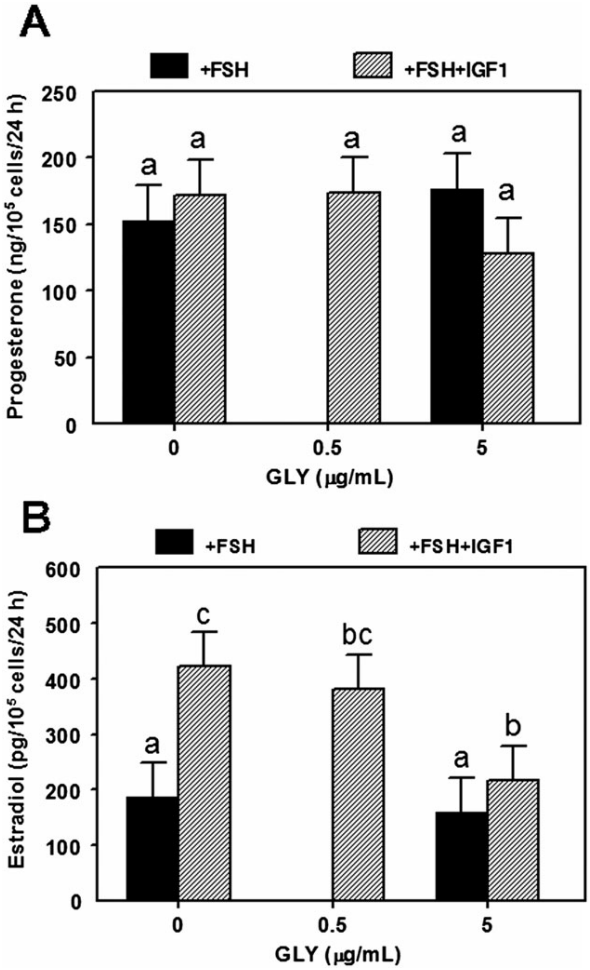


Figure 2. Effect of GLY on progesterone (A) and estradiol (B) production by granulosa cells from bovine follicles (experiment 1). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 48 h either with 30 ng/mL FSH alone and GLY at 0 or 5 µg/mL, or with 30 ng/mL FSH and IGF1 (30 ng/mL) with GLY at 0, 0.5 or 5.0 µg/mL. All cells were treated concomitantly with 500 ng/mL testosterone. Values are means +/-SEM from three separate experiments (n = 9). Within a panel, means without a common letter (a–c) differ (P<0.05). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.
(Figure adapted from Perego *et al.* (2017). Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*. Journal of Applied Toxicology 37:692-698. DOI 10.1002/jat.3417)

Experiment 2: Dose–response of glyphosate on granulosa cell proliferation and estradiol and progesterone production in the presence of follicle-stimulating hormone plus insulin-like growth factor 1
GLY at all tested concentrations (0.01 and 0.3 mg/mL) had no significant effect either on GC proliferation or steroid production. Cell numbers averaged 1.25, 1.47 and 1.00 ± 0.2 (×105 cells per well) for 0, 0.01 and 0.3 mg/mL GLY, respectively. P4 production averaged 99, 154 and 91 ± 23 ng 10-5 cells per 24 h for 0, 0.01 and 0.3 mg/mL GLY, respectively. E2 production averaged 130, 157 and 187 ± 28 pg 10-5 cells per 24 h for 0, 0.01 and 0.3 mg/mL GLY, respectively.

Experiment 3: Effect of glyphosate treatment on CYP19A1 and CYP11A1 mRNA in granulosa cells
The combined IGF1 plus FSH treatment increased (P<0.05) CYP19A1 and CYP11A1 mRNA abundance by threefold and twofold, respectively, above untreated control GC (Fig. 3A, B). GLY (5µg/mL) had no significant effect on CYP19A1 or CYP11A1 mRNA in GC co-treated with FSH and IGF1 (Fig. 3A, B).

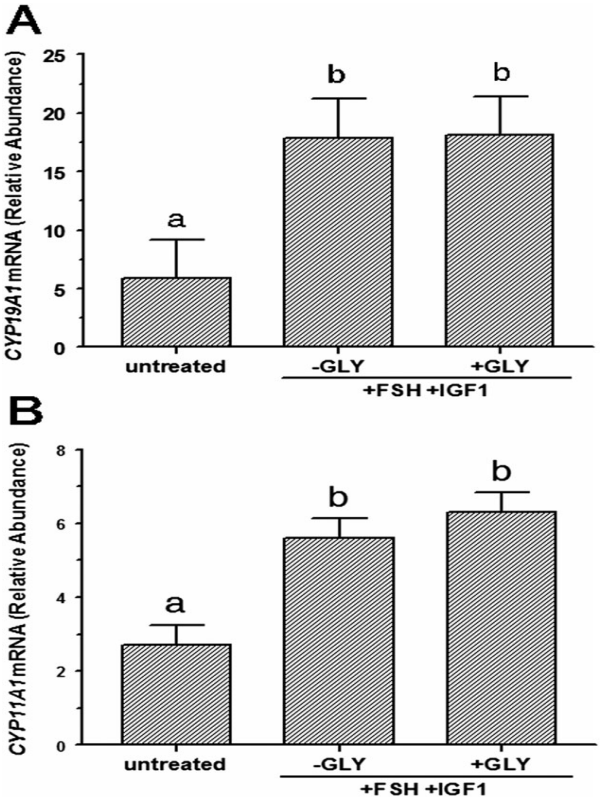


Figure 3. Effect of GLY on abundance of CYP19A1 (A) and CYP11A1 (B) mRNA in granulosa cells frombovine follicles (experiment 3). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 24 h either with either no additions (controls), or with FSH (30 ng/mL) and IGF1 (30 ng/mL) with GLY at 0 or 5.0 µg/mL. Values are means ± SEM from three separate experiments (n = 6). Within a panel, means without a common letter (a–b) differ ($P<0.05$). FSH, follicle stimulating hormone; GLY, glyphosate; IGF, insulin-like growth factor.
(Figure adapted from Perego *et al.* (2017). Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*. Journal of Applied Toxicology 37:692-698. DOI 10.1002/jat.3417)

Experiment 4: Dose–response of glyphosate on theca cell proliferation and androstenedione and progesterone production in the presence of luteinizing hormone with or without insulin-like growth factor 1

GLY at the tested concentration of 5 µg/mL had no significant effect on TC proliferation and P4 or A4 production in the presence of LH either with or without IGF1. IGF1 significantly increased cell numbers and steroid production. Cell numbers averaged 0.46, 0.53, 0.99 and 1.06 ± 0.05 for: LH alone; LH plus 5 µ/mL GLY; LH plus IGF1; and LH plus IGF1 plus 5 µ/mL GLY, respectively. P4 production averaged 40.5, 40.4, 70.3 and 78.3 ± 3.7 pg 10-5 cells per 24 h for: LH alone; LH plus 5 µg/mL GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg/mL GLY, respectively. A4 production averaged 1.45, 1.37, 2.75 and 2.47 ± 0.12 ng 10-5 cells per 24 h for: LH alone; LH plus 5 µg/mL GLY; LH plus IGF1 alone; and LH plus IGF1 plus 5 µg/mL GLY, respectively.

Experiment 5: Effect of glyphosate on serum-induced granulosa cell proliferation

Alone, GLY (1.7 µg/mL) increased ($P<0.05$) GC proliferation (Fig. 4). Cell numbers were increased by 11 % after 1 day and by 8 % after 2 days of GLY treatment (Fig. 4).

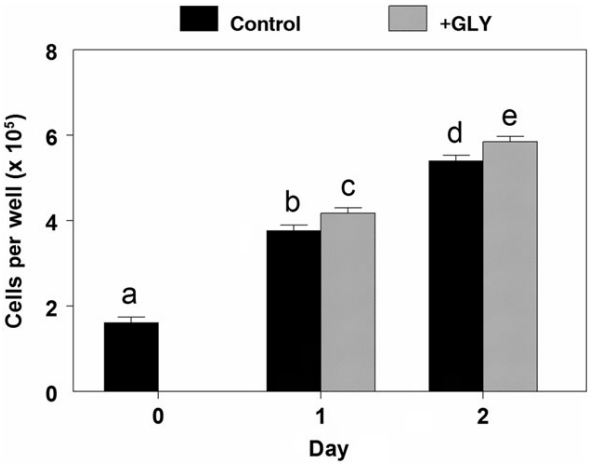


Figure 4. Effect of GLY on serum-induced proliferation of bovine granulosa cells (experiment 5). Cells were cultured for 48 h as described in Materials and methods, and then treated for an additional 0, 1 or 2 days with 10 % foetal calf serum and GLY at 0 or 1.7 µg/mL. Values are means ± SEM from three separate experiments (n = 9) Means without a common letter (a–e) differ (P<0.05). GLY, glyphosate.
(Figure adapted from Perego *et al.* (2017). Evidence for direct effects of glyphosate on ovarian function: glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells *in vitro*. Journal of Applied Toxicology 37:692-698. DOI 10.1002/jat.3417)

Conclusion

No effects were observed on GC P4 production in the presence of FSH either with or without IGF1, whereas GLY at 5 µg/mL had inhibitory effects on E2 production in the presence of FSH plus IGF1, showing a potential impairment on GC function that is essential for oocyte survival (Petro *et al*., 2012). Results of study also showed that GLY at 5 µg/mL had an inhibitory effect on GC proliferation in the presence of FSH plus IGF1, and this inhibitory effect of 5 µg/mL GLY on cell numbers was not associated with a change in cell viability.

Assessment and conclusion

Assessment and conclusion by applicant:

In this *in vitro* study, glyphosate had minimal effects on granulosa cells (GC). In the presence of FSH only, glyphosate had no effect on GC cell viability or on progesterone or estradiol production. In the presence of FSH and IGF1, glyphosate reduced GC proliferation without a dose-response at 0.5 and 5 µg/mL but not at lower test concentrations (0.01 and 0.3 µg/mL) and did not affect progesterone production or CYP19A1 and CYP11A1 mRNA expression; estradiol production was reduced at 5 µg/mL only (not at lower test concentrations). Without FSH or IGF1, 1.7 µg/mL of glyphosate slightly increased GC proliferation in response to serum (≤11 %).

Glyphosate at 5 µg/mL had no effect on the theca cell (TC) proliferation or the production of progesterone or androstenedione.

Overall, with the exception of slight, non-dose-related alterations in GC proliferation under different test conditions, this study showed no effects of glyphosate on GC at physiologically relevant test concentrations. Glyphosate had no effect on TH.

This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterised, no positive controls were used and the tests were conducted with only one or 2 test concentrations of glyphosate.

Reliability criteria made by the applicant

Publication: Perego *et al.,* 2017	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	

Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity not reported. Source: Sigma Aldrich.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Test system clearly and completely described	Y	Bovine granulosa and theca cells.
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	Not applicable	
Test concentrations in physiologically acceptable range (< 1 mM)	Y (partly)	0, 0.5, 5, or 0, 10, 300 µg/mL (1.77 mM), or 0, 5 µg/mL.
Cytotoxicity tests reported	Y?	Viability tested at only one concentration of glyphosate.
Biochemical methods described	Y?	Some could be more detailed.
Positive and negative controls	N	No positive controls.
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	Y	Limited since max. 2 test concentrations.
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Reliability not assignable		
Not reliable		
This publication is considered relevant for the risk assessment of glyphosate but reliable with restrictions because the glyphosate tested was not sufficiently characterised, no positive controls were used and the tests were conducted with only one or 2 test concentrations of glyphosate.		

Assessment and conclusion by RMS:

In this study effects of glyphosate on ovarian cell proliferation, steroid production and gene expression were evaluated using bovine granulosa cells (GC) and theca cells (TC) as in vitro models. Glyphosate at 5 µg/mL had no effect on the theca cell proliferation, nor on Cyp19A1 and Cyp11A1 mRNA in granulosa cells. No effects were observed on GC progesterone (P4) production in the presence of follicle-stimulating hormone (FSH) either with or without insulin-like growth factor (IGF1), whereas Glyphosate at 5 µg/ml had inhibitory effects on estradiol (E2) production in the presence of FSH plus IGF1, showing a potential impairment on GC function under the circumstances of this *in vitro* study.

The study is relevant for risk assessment as the endpoints examined inform on the female reproductive function. The study is reliable with restrictions because the glyphosate used is not sufficiently characterised, no positive controls were used and the tests were conducted with few test concentrations.

Category A – Dai, P. *et al.*

Data point	CA 5.6.1/023
Report author	Dai, P. *et al.*
Report year	2016
Report title	Effect of glyphosate on reproductive organs in male rat
Document No.	doi.org/10.1016/j.acthis.2016.05.009

	E-ISSN: 1618-0372
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, in Addendum (2017) to RAR on ED properties
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study
In this study SD rats were lavaged with glyphosate at doses of 5, 50, 500 mg/kg to detect the toxicity of glyphosate on rat testis.

Materials and methods

Test substance	Isopropylamine salt of glyphosate (90 % w/w purity) was purchased from Shanghai Ryon Biological Technology Co. Ltd., China.
Lot/Batch	Not specified
Purity/Radiochemical purity	90 % w/w
Animals	Animals - 32 sexually mature 56–day old Sprague-Dawley (SD) male rats were raised in an animal house and maintained in an air-conditioned room at approx. 21 °C with a 12 h/12 h light/dark cycle. A balanced mixture of pelleted food and water were available to the rats.
Experimental design and treatment	32 rats were randomly divided into 4 groups, 3 groups were orally given glyphosate as an aqueous solution by gavage once a day. The control group was treated in the same way with deionised water. The doses administered were 5, 50, and 500 mg/kg bw. All rats were treated for 5 weeks continuously. After the last treatment the rats were sacrificed and testis, epididymis, prostate gland and seminal vesicle removed and weighed.
Epididymal sperm parameters	Epididymal sperm was used for the measurement of total sperm count. The epididymis was minced in PBS and filtered using a nylon mesh screen. The filtrate was treated with 10 mL PBS and the number of sperm was counted using a standard hemo-cytometric method.
Hormone measurement	Serum hormones were measured by radioimmunoassay using a ^{125}I-labeled ligand double-antibody RIA Kit for total testosterone, estradiol and progesterone. The minimum sensitivity of the method was 0.02 ng/mL for testosterone, less than 0.2 ng/mL for progesterone and less than 5 pg/mL for estradiol. The intra- and inter-assay coefficients of variation (CV) were less than 10 %.
Testicular, epididymal and seminal vesicle gland histology	Following fixation of the tissues, the samples were passed through a graded series of ethanol and xylene solutions and embedded in paraffin wax. Paraffin-embedded tissues were serially sectioned at 5 µm thickness. For each rat, two non-serial sections were stained with hematoxylin/eosin (HE).
Antioxidant status analysis	The levels of catalase (CAT, U/mg protein), superoxide dismutase (SOD, U/mg protein) and malondialdehyde (MDA, µmol/g protein) were determined by the absorbance of samples in multiskan spectrum. SOD activity was determined by an SOD assay kit with absorbance measured at 560 nm. CAT activity was determined by the H_2O_2 consumption (µmol/g protein) with absorbance measured at 405 nm. Lipid peroxidation was determined by measurement of MDA by the TBA test with absorbance at 532 nm.
Immunohistochemistry	Sections of the testes were deparaffinised with xylene and rehydrated in graded ethanol before being washed with twice-distilled water. To increase epitope exposure, the sections were heated for 15 minutes in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven. The sections were then cooled and washed with 0.01 M PBS at pH 7.2 and then blocked with 10% bovine serum albumin (BSA) in TBST (20 mM Tris-buffered saline, 0.05 % Tween 20, pH 7.5) for 1 hour at room temperature. The sections were incubated overnight at 4oC with diluted (1:400) polyclonal antibodies against androgen receptor (N-20; rabbit anti-human AR). The secondary antibody was goat

	anti-rabbit IgG. The binding of the antibodies were visualised using a SABC Kit Elite and 0.05 % 3,3-diaminobenzidine tetrachloride in 0.01 M PBS at pH 7.2, containing 0.01 % H_2O_2 for 2 minutes. The sections were counter stained with hematoxylin and mounted with cover slips. The specificity of the antibody was examined using 1 % BSA rather than the primary antibody.
Data analysis	All results are means ± SEM. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Tukey's multiple comparison test. Significant differences with controls were considered when $p<0.05$.

Results

Average daily weight gain and average daily feed intake - Daily exposure to glyphosate caused a statistically significant decrease in average daily feed intake at 50 mg/kg bw/day only. Although not statistically significant there was a dose-dependent decrease in daily weight gain.

Table 1
Body weights and organ weights of male rats treated with glyphosate after 5 weeks gavage.

	Control	Glyphosate (mg/kg body weight)		
	0	5	50	500
Body weight (g)	388.60 ± 7.08	404.00 ± 5.71	351.50 ± 20.85	351.80 ± 7.74
Testis(g)	3.10 ± 0.11	3.04 ± 0.10	2.59 ± 0.22	3.06 ± 0.19
Relative testis(%)	0.83 ± 0.05	0.75 ± 0.02	0.73 ± 0.05	0.87 ± 0.04
Epididymis (g)	1.09 ± 0.04	1.13 ± 0.03	0.98 ± 0.09	1.07 ± 0.05
Relative epididymis(%)	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.02	0.30 ± 0.01
Prostate(g)	0.45 ± 0.03	0.45 ± 0.02	0.39 ± 0.06	0.40 ± 0.05
Relative prostate(%)	0.12 ± 0.01	0.11 ± 0.01	0.011 ± 0.01	0.011 ± 0.01
Seminal vesicle gland and coagulating gland (g)	1.58 ± 0.25[a]	1.49 ± 0.20[ab]	1.23 ± 0.48[ab]	1.10 ± 0.29[b]
Relative Seminal vesicle glandand coagulating gland (%)	0.42 ± 0.02	0.37 ± 0.02	0.34 ± 0.04	0.31 ± 0.03

Data are means ± SEM (n = 8,8,8,6).
The characters indicate significant differences between the compared groups ($p< 0.05$).

(Table adapted from Dai, P. *et al.* (2016). Effect of glyphosate on reproductive organs in male rat. Acta Histochemica 118 519-526 doi.org/10.1016/j.acthis.2016.05.009)

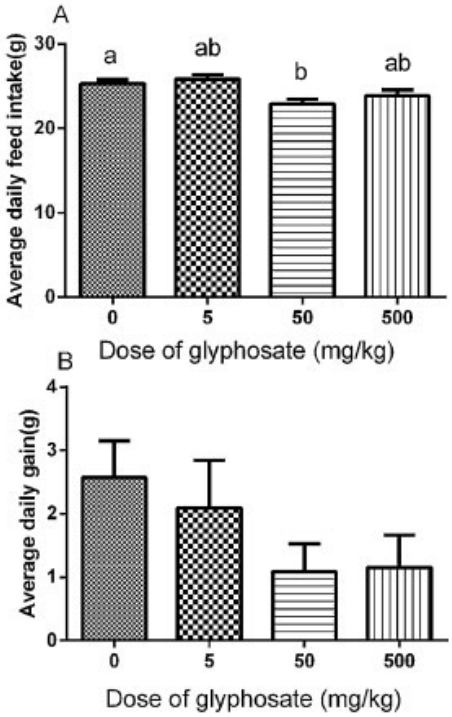


Fig. 1. Average daily feed intake (A) and average daily gain (B). The values shown are the means ± SEM (n = 5). The characters indicate significant differences between the compared groups ($p< 0.05$).

(Figure adapted from Dai, P. *et al.* (2016). Effect of glyphosate on reproductive organs in male rat. Acta Histochemica 118 519-526 doi.org/10.1016/j.acthis.2016.05.009)

Reproductive organ weights and sperm parameters - Seminal vesicle and coagulating gland absolute weight showed a statistically significant changes amongst treatment groups whereas no such change was observed in other reproductive organs. No significant differences were observed in relative reproductive organ weights. Total sperm count was statistically significantly decreased at 500 mg/kg bw.

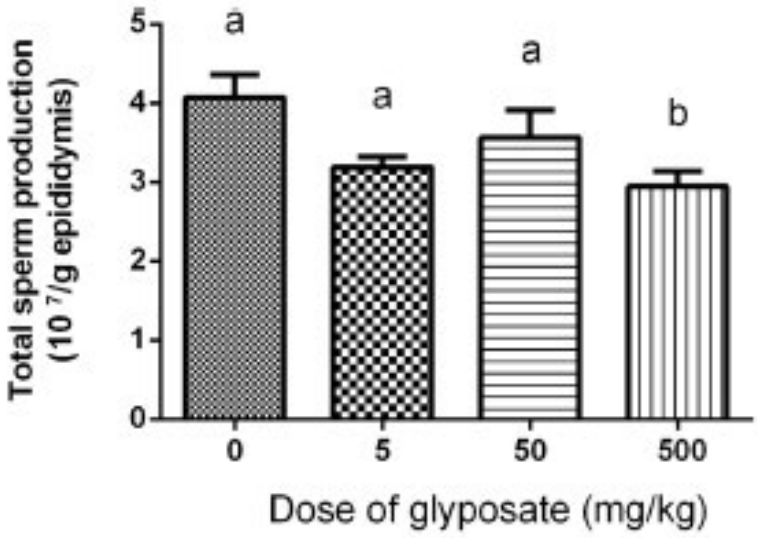


Fig. 2. Effects of glyphosate on total sperm count. Values are means ± SEM (n = 8,8,8,6). The characters indicate significant differences between the compared groups ($p < 0.05$).

(Figure adapted from Dai, P. *et al.* (2016). Effect of glyphosate on reproductive organs in male rat. Acta Histochemica 118 519-526 doi.org/10.1016/j.acthis.2016.05.009)

Concentrations of testosterone, estradiol and progesterone in serum - Although there was a trend towards decreased serum concentrations with dose for testosterone and progesterone no statistically significant changes were noted in the serum concentrations of testosterone, estradiol and progesterone.

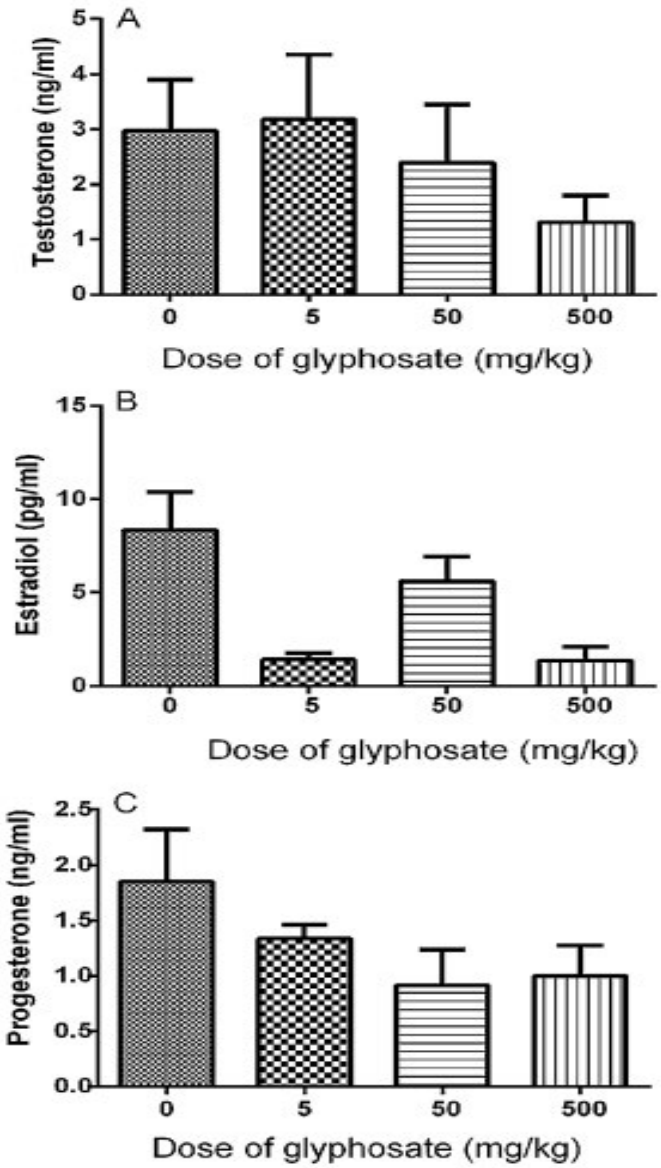


Fig. 3. The level of steroid hormones in serum of glyphosate-treated rats. Testosterone (A), estradiol (B), progesterone (C). Values are means ± SEM (n = 8,8,8,6).

(Figure adapted from Dai, P. *et al.* (2016). Effect of glyphosate on reproductive organs in male rat. Acta Histochemica 118 519-526 doi.org/10.1016/j.acthis.2016.05.009)

SOD and CAT activity, H2O2 and MDA levels in testes - There were no statistically significant changes in SOD and CAT activity and H2O2 and MDA levels in testes.

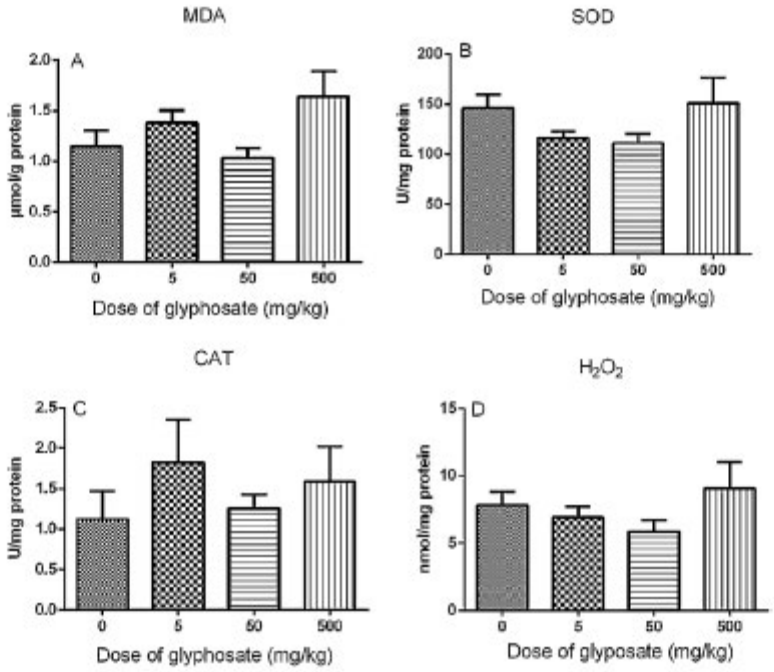


Fig. 4. The effects of glyphosate on MDA level (A), SOD activity (B), CAT activity (C), and H_2O_2 level (D) in rats testis. Values are the means ± SEM (n= 8,8,8,6).

(Figure adapted from Dai, P. *et al.* (2016). Effect of glyphosate on reproductive organs in male rat. Acta Histochemica 118 519-526 doi.org/10.1016/j.acthis.2016.05.009)

Testicular, epididymal and seminal vesicle gland histology - No statistically significant changes were observed in the histopathology of the testis, epididymis and seminal vesicles.

Immunohistochemical localisation of androgen receptor in the testis - No statistically significant changes were observed in androgen receptor (AR) immunoreactivity localised in the nuclei of cells, including Sertoli cells, peritubular myoid cells and Leydig cells.

Discussion and conclusion

The present study provides information on the potential effects of glyphosate on the reproductive system of the male rat. Average daily weight gain showed no substantial decrease whereas average daily feed intake was significantly decreased at 50 mg/kg bw but not at 500 mg/kg bw. It is therefore suggested that the decrease of average daily feed intake is independent of glyphosate treatment. Although there are no statistically significant differences in average weight gain, the trend is a decrease. At 500 mg/kg bw the absolute weight of seminal vesicle gland and coagulating gland and total sperm count decreased substantially. There was no significant change in oxidative stress parameters after oral administration of glyphosate at doses up to 500 mg/kg bw. Testosterone, estradiol and progesterone serum levels, as well as AR in testis presented no significant changes when compared to controls.

Assessment and conclusion

Assessment and conclusion by applicant:

The potential toxicity of glyphosate to the male reproductive system of the rat has been investigated after oral treatment with glyphosate for 5 weeks at dose levels up to 500 mg/kg bw. The endpoints studied were body weight, food intake, daily weight gain, absolute and relative reproductive organ weight, serum hormone levels, oxidative stress parameters, testicular histopathology and expression of AR in testis. The effects found were a significant decrease in absolute (but not relative) weight of the seminal vesicle gland and coagulating gland and a decrease in sperm count at the highest dose tested.

This publication is considered relevant but reliable with restrictions because there are deviations from regulatory guidelines for reproductive toxicity studies and the reproductive effects seen are not corroborated by the results from guideline studies at similar dose levels.

Reliability criteria made by the applicant

Publication: Dai *et al*., 2016	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	Incomplete study
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	Y	Purity of 90 % as isopropylamine salt. Source Shanghai Ryon Biological Technology Co. Ltd., China.
Only glyphosate acid or one of its salts is the tested substance	Y	Isopropylamine salt
AMPA is the tested substance	N	
Study		
Test species clearly and completely described	Y	
Test conditions clearly and completely described	Y?	
Route and mode of administration described	Y	
Dose levels reported	Y	
Number of animals used per dose level reported	Y	8 males per group
Method of analysis described for analysis test media	N	
Validation of the analytical method	N	
Analytical verifications of test media	N	
Complete reporting of effects observed	Y?	
Statistical methods described	Y	
Historical control data of the laboratory reported	N	
Dose-effect relationship reported	Y	Results are not concordant with outcome of regulatory reproduction toxicology studies
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant but reliable with restrictions because there are deviations from regulatory guidelines for reproductive toxicology studies and the reproductive effects seen are not corroborated by the results from regulatory studies at similar dose levels.		

Assessment and conclusion by RMS:

The effects found were a significant decrease in absolute (but not relative) weight of the seminal vesicle gland and coagulating gland noted for all treated groups, and a decrease in sperm count at the highest dose tested (500 mg/kg). It could also be noted that there was a trend towards decreased serum concentrations with dose for testosterone and progesterone. The magnitude of decreased weight of seminal vesicle gland and coagulating gland was 22% and 30% at mid and high dose level, respectively, but this effect was only accompanied by decreased sperm count at the highest dose level. Testicular, epididymal and seminal vesicle gland histology showed no significant differences compared to controls. It could be noted that reduced body weight was observed at mid- (10%) and high dose level (9%), although not statistically significant. The study is relevant for risk assessment as the endpoints examined inform on the male reproductive function.

The study is no GLP study. Limited parameters were investigated in the study, low number of animals used, and no details about clinical signs. The use of this study for regulatory purposes is limited.

Category A – Forgacs, A.L. *et al.*

Data point	CA 5.6.1/024
Report author	Forgacs, A.L. *et al.*
Report year	2012
Report title	BLTK1 Murine Leydig Cells: A Novel Steroidogenic Model for Evaluating the Effects of Reproductive and Developmental Toxicants
Document No.	Toxicological Sciences 127(2), 391–402 doi:10.1093/toxsci/kfs121 E-ISSN: 1096-0929
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Yes, in Addendum (2017) to RAR on ED properties
GLP/Officially recognised testing facilities	Non-GLP
Acceptability/Reliability	Yes/Reliable with restrictions

Aim of the study

The aim of this study was to evaluate the effect of several structurally diverse endocrine disrupting compounds (EDCs) on steroidogenesis in a novel BLTK1 murine Leydig cell model.

Materials and methods

Chemicals	Origin of the glyphosate sample tested was not reported.
Lot/Batch	Not specified
Purity/Radiochemical purity	Not specified
Cell culture and treatment	Mouse Leydig BLTK1 (BLT-1 cells, clone K1) cells were isolated from a testicular tumor that developed in a transgenic mouse expressing the mouse inhibin α promoter/simian virus 40 T-antigen fusion gene. Cells were maintained in phenol red–free DMEM/F-12 media with 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin and incubated in 5 % CO_2 at 37 °C). For the evaluation of steroidogenic enzyme and receptor expression, cells were grown to 80% confluency and harvested without any treatment. For the determination of 3',5'-cyclic adenosine monophosphate (cAMP), progesterone (P), testosterone (T), and estradiol (E2), cells were grown to 80% confluency, transferred into 24-well tissue culture plates and incubated overnight. Cells were treated with DMSO, or with 0.1, 0.3, 1, 3, 10, 30, or 100 ng/mL recombinant human chorionic gonadotropin (rhCG) or with 0.1, 0.3, 1, 3, 10, 30, or 100 µM Forskolin (FSK) and media were collected at indicated times. Time course studies were conducted with DMSO, 3 ng/mL rhCG or 10 µM FSK, and media were collected after 1, 2, 4, 8, 12, 24, or 48 hours of incubation. Gene expression studies used the same study design, concentrations and time points with cells seeded into T-25 flasks.
MTT assay	BLTK1 cells placed in 96-well plates were treated with 1, 3, 10, 30, 100, 300, or 600 ng/mL rhCG, 1, 3, 10, 30, 100, 300, or 600 µM FSK or 1, 3, 10, 30, 100, 300, or 600 µM of test compound in triplicate. Media were aspirated after 24 hours and replaced with 50 µL of fresh MTT reagent (5 mg/mL thiazolyl blue tetrazolium bromide in PBS). Following 3 hours of incubation, MTT reagent was removed and replaced with 150 µL DMSO. Cells were incubated for 2 hours followed by absorbance measurements at 595 and 650 nm using an Emax precision microplate reader. Results are reported as percentage of control calculated from the relative absorbance of treated versus DMSO controls where 100 % indicates no cytotoxicity.
RNA isolation and gene expression	Total RNA was extracted from cell pellets using RNeasy Mini Kits with an additional RNase-free DNase digestion. RNA was quantified at 260 nm and purity assessed using the A260/A280 ratio, as well as by denaturing gel electrophoresis. First-strand complementary DNA (cDNA) was synthesized from RNA (1 µg) using SuperScript II reverse transcriptase and anchored oligo-dT primer. For real-time PCR (RT-PCR) evaluation of steroidogenic enzyme and receptor expression, cDNA was used as a template for PCR amplification with gene-specific primers. Quantitative RT-PCR (QRT-

	PCR) was used to quantify concentration- and time-dependent expression of specific genes. Reactions in 96-well plates consisted of 30 µL, including 1 µL of cDNA template, 0.1 µM forward and reverse gene-specific primers using an Applied Biosystems PRISM 7500 Sequence Detection System. Dissociation curve analysis assured single product amplification. To control for differences in RNA loading, quality and cDNA synthesis, samples were standardised to the geometric mean of three housekeeping genes: *ActB*, *Gapdh*, and *Hprt*. Results were quantified using a standard curve generated on the same 96-well plate and amplified by using purified cDNA product as template specific for each gene (serial 10-fold dilutions from 10^8 to 10^1 copies). The slope of the standard curve was used to assess amplification efficiency with all amplification efficiencies >90 %. Fold changes were calculated relative to time-matched vehicle. Relative expression was scaled such that time-matched vehicle control expression equaled one for graphing purposes.
Dose-response modeling and statistical analyses	The ToxResponse modeler uses particle swarm optimisation to identify the best fit across five model classes: sigmoidal, exponential, linear, quadratic, and Gaussian. The best fitting model was then used to calculate half maximal effective concentration (EC_{50}) values. All statistical analyses were carried out using SAS v9.1 by ANOVA, with Dunnett's or Tukey's *post hoc* tests for concentration-response and time course data, respectively. Differences between treatment groups were considered significant when $p<0.05$ relative to time-matched DMSO control.

Results

Steroidogenic Enzyme Expression in BLTK1 Cells - Steroidogenic enzyme messenger RNA (mRNA) and protein were detected in BLTK1 cells by RT-PCR and/or Western blotting, confirming the expression of all required steroidogenic enzymes. In addition, mRNA for several potential regulatory factors including LHCGR, estrogen receptor (ER), androgen receptor (AR), and steroidogenic factor 1 (SF-1), peroxisome proliferator-activated receptors (PPARa and PPARc), the pregnane X receptor (PXR), and the aryl hydrocarbon receptor (AhR) were also detected. However, mRNA for progesterone receptor, glucocorticoid receptor, or the liver receptor homolog 1 was not detected in BLTK1 cells despite verification of RT-PCR primer specificity and functionality in mouse Hepa1c1c7 cells.

Induction of Steroidogenesis by FSK and rhCG - Temporal profiles of intracellular cAMP as well as P and T levels in media were evaluated in response to 3 ng/mL rhCG or 10 µM FSK by enzyme immunoassay (EIA). Intracellular cAMP was induced by FSK after 30 minutes (~120 pmol/mL, ~10-fold) and after 1 hour in response to rhCG (635 pmol/mL, 60-fold). However, levels quickly diminished such that no intracellular cAMP was detected by 8 hours. Maximum P levels (200 ng/mL, 8-fold) were observed after 2 hours in response to rhCG and FSK, followed by a steady decline due to metabolism to androgens and estrogens. In contrast, T levels gradually increased reaching a maximum of ~200 pg/mL (7-fold) after 48 hours, with significant increases as early as 1 hour post treatment. Concentration-dependent induction of intracellular cAMP and secreted P and T was evaluated after 4 hours when cAMP could still be detected. 17β-Estradiol (E2) was evaluated after 48 hours as it was not consistently detected after 4 hours. cAMP, P, and T were induced 25-, 10-, and 4-fold, respectively, after 4 hours, whereas E2 was induced ~4-fold by 48 hours. The EC50 for cAMP induction was greater than 24 ng/mL for rhCG and greater than 29 µM for FSK. Meanwhile, EC50 values of 1 ng/mL rhCG and 9 µM FSK were conserved for both P and T induction, whereas E2 EC50 values were 10 ng/mL for rhCG and 9 µM for FSK. Intracellular cAMP levels are not only regulated by synthesis but also by degradation, which is regulated by cyclic nucleotide phosphodiesterase enzymes. The phosphodiesterase inhibitor IBMX maximizes cAMP levels in order to further induce steroidogenesis. However, IBMX co-treatment with rhCG or FSK did not increase T levels further, albeit rhCG and FSK potencies were greater with and without IBMX by FSK: 0.1 µM vs. 9.4 µM vs. 0.9 ng/mL, respectively. When tested in this system glyphosate at 300 µM did not induce T production nor alter rhCG induction of T.

Conclusion

Current test protocols and models are inadequate to screen the universe of chemicals, metabolites, and mixtures that may alter steroidogenesis. BLTK1 cells are a novel complementary rhCG-inducible Leydig-based model that can be used to assess effects on steroidogenic gene expression, intracellular cAMP, and P, T, and E2 levels in media. Their consistent response characteristics and inducibility over 30 passages also make this cell line attractive for high-throughput screening. Comprehensive characterisation of effects on intermediate steroid biosynthesis, including pregnenolone, 17-hydroxyprogesterone, DHEA, androstenedione, estrone, and DHT, as

well as the differential expression of steroidogenic enzymes will also facilitate the elucidation of modes of action relevant to adverse outcome pathways in humans and other relevant species.
When tested in this system glyphosate at 300 µM did not induce testosterone production or alter rhCG induction of testosterone.

Assessment and conclusion

Assessment and conclusion by applicant:
In this study, recombinant human chorionic gonadotropin (rhCG) and forskolin (FSK) were used as positive controls for the induction of steroidogenesis, as measured by increases in progesterone, testosterone and 17β-estradiol levels in culture media. Murine BLTK1 Leydig cells were investigated as a novel model for evaluating the effects of chemicals on steroidogenesis. The results demonstrated that BLTK1 cells can be used to screen substances that alter intracellular cAMP, steroidogenic gene expression, and sex steroid levels. When tested in this system glyphosate was not found to induce testosterone production or alter rhCG induction of testosterone.

This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the test substance was not characterised and the results of only one concentration level were reported.

Reliability criteria made by the applicant

Publication: Forgacs *et al*., 2012	Criteria met? Y/N/?	Comments
Guideline-specific		
Study in accordance to valid internationally accepted testing guidelines	N	
Study performed according to GLP	N	
Study completely described and conducted following scientifically acceptable standards	Y?	
Test substance		
Test material (Glyphosate) is sufficiently documented and reported (i.e. purity, source, content, storage conditions)	N	Purity and source not reported.
Only glyphosate acid or one of its salts is the tested substance	Y	
AMPA is the tested substance	N	
Study		
Test system clearly and completely described	Y	
Test conditions clearly and completely described	Y	
Metabolic activation system clearly and completely described	N	
Test concentrations in physiologically acceptable range (< 1 mM)		
Cytotoxicity tests reported	Y	
Positive and negative controls	Y	
Complete reporting of effects observed	Y	
Statistical methods described	Y	
Historical negative and positive control data reported	N	
Dose-effect relationship reported	N	Only one concentration was tested (300 µM) Glyphosate, did not induce or alter rhCG induction of T. Glyphosate also had no effect on T levels in BLTK1 cells
Overall assessment		
Reliable without restrictions		
Reliable with restrictions	Y	
Not reliable		
This publication is considered relevant for glyphosate risk assessment but reliable with restrictions because the test substance was not characterised and only one concentration level was tested.		

Assessment and conclusion by RMS:
In this study, it was demonstrated that BLTK1 cells can be used to screen substances that alter intracellular cAMP, steroidogenic gene expression, and sex steroid levels. When tested in this system glyphosate was not found to induce testosterone production or alter rhCG induction of testosterone.

The study is reliable with restrictions because the test substance was not characterised and the results of only one concentration level were reported.

Summary of published literature studies identified by the applicant as supplementary after detailed assessment of full-text articles (Category B)

Category B – Sakpa, C.L. *et al.*

Data point	CA 5.6
Report author	Sakpa C. L. *et al.*
Report year	2018
Report title	Effects of glyphosate on sperm parameters and pregnancy success rate in Wistar rats
Document source	Annals of Biomedical Sciences (2018), Vol. 17, No. 2, pp. 156-164
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary

Aim of the study
The aim of the study was to explore the impact of glyphosate on the characteristics of spermatozoa and pregnancy success rate in females following oral administration of glyphosate in male rats. The specific objectives include the examination of the effects of glyphosate on sperm count, motility and morphology in adult Wistar rats and to determine the pregnancy success rates and litter size in females mated with glyphosate treated male Wistar rats.

Materials and methods

Test material:	Glyphosate marked as "Tackle" and manufactured by The Candel FZE F-08-004, along N4-E7 road, Lekki, Lagos, Nigeria
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Deionized water
Test animals:	Rat
Strain:	Wistar
Age/weight on arrival:	250 g to 300 g / Age not reported
Source:	Animal Holding on the Department of Anatomy, University of Benin
Housing:	Not reported
Acclimatisation period:	2 weeks
Diet:	Rat chow, *ad libitum*
Water:	Not specified, *ad libitum*
Environmental conditions:	Temperature, light/dark cycle and humidity not reported

Animal procurement and experimental design
Thirty adult Wistar rats comprised of 15 males and 15 females weighing between 250 and 300 g were used for this study. The male rats were randomly categorized into three groups; A, B and C with 5 rats in each group.

Group A represented the control rats and was treated with 1 mL daily of distilled water. Group B and C rats were treated with 400 and 2000 mg/kg bw/day of glyphosate dissolved in 1 mL of distilled water, respectively. Dosages were based on oral LD50 of glyphosate in rats which is 5600 mg/kg bw. Rats in all the groups were treated for 60 days based on the spermatogenic cycle of Wistar rats. At day 61 of the experiment, 5 female rats were randomly selected and incorporated into each established male group. A pair of male and female rats from each group were housed in separate cages and mated to evaluate pregnancy success rate. Mating was done after ensuring that the female rats were in their oestrous phase of the menstrual cycle. Pregnancy was confirmed by the presence of spermatozoa in a vaginal smear observed with a microscope the morning after mating.

Upon ensuring successful mating each male rat was then sacrificed by cervical dislocation following chloroform anaesthesia. The abdominopelvic cavity was entered through a ventral midline incision. The ductus deferens was identified, isolated and quickly ligated to a length of about 30 mm minimum at either end. Semen analysis was carried out according to established standard laboratory protocol. Semen from the ductus deferens was released into 1 mL of normal saline (0.9% NaCl) in a 10 mL plastic universal container and the suspension agitated gently. Two drops of sperm suspension were smeared on a glass slide using a micropipette and two drops of warm 2.9% sodium citrate were added. A coverslip was then applied and the suspension examined under the microscope using X100% oil immersion objective for sperm motility. The total sperm count was calculated using Neubauer haemocytometer. Sperm cells were taken from the original dilution for motility, diluted 1:20 with 10 % neutral buffered formalin, and evaluated with aid of light microscope at X100 magnification. Five hundred sperm cells from the sample were scored for morphological abnormalities. Normal sperm cell had a head, neck and tail while abnormal sperm cells had one or more of the following features: deformed head, headless tail, tailless head, fatty deposit, degenerated sperm particles and conjoined spermatozoa. Abnormal cells were expressed as a percentage of morphologically normal sperm. The slides were examined under a microscope and photographs were taken for preparation of photomicrographs.

Data analysis
The data on sperm motility, morphology and sperm count, as well as the effects of treatment on pregnancy and litter size, were collated and entered into Statistical Package for Social Scientists (SPSS version 16) software for t test and test of significance. The level of statistical significance was set at $p < 0.05$. The results were represented in words, tables and figures using Microsoft Office software.

Results

Sperm parameters
Table 1 shows the results for sperm parameters upon glyphosate exposure. Progressive motility and total sperm count were decreased compared to control, reaching statistical significance in the high dose group. However, non progressive motility and immobility were increased compared to control. These increases were dose dependent but not statistically significant. Abnormal morphology was significantly increased and showed dose dependency. The rats in the control group showed normal sperm morphology while the spermatozoa of rats in the treated groups showed varying degrees of sperm abnormalities including; headless tails, tailless heads, conjoined sperm cells, swollen heads and generated sperm particles.

Table 1*: Sperm characteristics of male Wistar rats

	(Sterile water) n=5	(400 mg/kg/day Glyphosate) n=5	2000 mg/kg/day Glyphosate n=5
Progressive Motility	70.00±4.47	64.00±5.10	46.00±4.00*
Non-Progressive Motility	12.00±2.00	16.00±4.00	24.00±7.48
Immobility	18.00±3.74	20.00±5.48	30.00±8.94
Total Sperm Count	323.60±21.22	280.60±25.76	193.60±4.75*
Normal morphology	91.00±1.00	74.00±2.45*	34.00±7.48*
Abnormal morphology	9.00±1.00	26.00±2.45*	66.00±7.48*

Data are represented as Mean ± SEM, n=5; * represents significant (P<0.05) difference compared with control group.

*Retrived from Sakpa, C.L., *et al*., Ann Biomed Sci Vol 17, No 2, June 2018, pp. 156-164

Pregnancy success rate following mating of male and female rats

The pregnancy success rate was 40% and 20% respectively for low and high dose treatment groups compared to that of the control of 100% success rate. The mean litter size was 1.8 and 0.6 for low and high dose groups, respectively, while that for the control was 8.2 (Table 2). The findings indicate a significant difference in the mean litter size in both treatment groups compared to control.

Table 2: Pregnancy success rate of female Wistar rats

Parameter	Control group (Sterile water) (n= 5)	Low dose group (400mg glyphosate/kg/day) (n= 5)	High dose group (2000mg glyphosate/kg/day) (n= 5)
Number of pregnant rats	5.00	2.00	1.00
Litter size	8.20 ± 0.37	1.80 ± 1.11*	0.60 ± 0.6*
% pregnancy outcome	100.0	40.0	20.0

Litter size is represented in Mean ± SEM, n=5; * represents significant *(P<0.05)*difference compared with control group.

*Retrived from Sakpa, C.L., *et al*., Ann Biomed Sci Vol 17, No 2, June 2018, pp. 156-164

Conclusion

In the present study, glyphosate caused a dose dependent progressive decline in sperm count and motility indices of treated rats. The injurious effect of glyphosate on sperm morphology amongst the two treated groups manifested as greater than 10 % abnormal forms. These abnormal forms included headless tails, tailless heads, conjoint sperm cells, swollen heads and degenerated sperm particles which were observed amongst the high dose group, thereby confirming the dose dependent effects of glyphosate. These findings suggest that the treated rats had been rendered infertile by exposure to glyphosate. To further confirm the antifertility effect, glyphosate treated male rats were used to mate untreated females, and the outcome showed decreased pregnancy success rates to 20% with a concomitant reduction in litter size among the high dose groups.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: The glyphosate used was not sufficiently characterized and the overall composition tested and administration methods are unknown. This makes a comparison to the representative EU test substance and formulations not possible. Only two dose levels were tested and the number of animals used per dose level was too low. Additionally, no details were provided on how the animals were housed or reared in the study and whether any steps were taken to consider animal welfare. Number of animals not sufficient (5 per sex and group). Administration method not clearly stated (gavage suspected). This publication is considered unreliable.

The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Further points for clarification:
The study aimed to investigate reproductive performance and sperm quality upon administration of a glyphosate based herbicide to rats for 60 days at 400 or 2000 mg/kg bw. A dose dependent and significant effect was observed in the treated rats concerning decreased litter size, decreased progressive motility of sperm, decreased total sperm count and increased abnormal sperm morphology. These findings suggest that glyphosate causes infertility in males when treated with the glyphosate based herbicide "Tackle".

Assessment and conclusion by RMS:
In this study, groups of 5 male Wistar rats received glyphosate-based herbicide "Tackle" by gavage at concentrations of 400 or 2000 mg/kg bw/day for 60 days. Male rats in control group received treatment with 1 ml of distilled water daily. As from day 61 of the experiment male rats from each group were allowed to mate with females in their groups. The rats were allowed to litter and the pregnancy success rate and litter size documented. Male rats were then sacrificed and semen collected from the ductus deferens for motality, morphology and sperm count studies. Decreased pregnancy success rate was observed in low (40%) and high (20%) dose treatment groups compared to 100% success rate for the control group. Concomitant reduction in litter size were observed among the treatment groups. The result of the sperm investigations showed decreased sperm count and motility and abnormal morphology in treated groups.

The study is considered as supplementary data. The study is reliable with restrictions because of the following reasons: the test substance is not sufficiently characterised, only two doses tested, small group sizes, clinical observations not presented, body weights not recorded.

Category B – Cai, W. *et al.*

Data point	CA 5.6
Report author	Cai W. *et al.*
Report year	2017
Report title	Effects of glyphosate exposure on sperm concentration in rodents: A systematic review and meta-analysis
Document source	Environmental Toxicology and Pharmacology (2017), Vol. 55, pp. 148-155
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	Not applicable
Acceptability/Reliability	Supplementary

Aim of the study

The aim of this study was to explore the potential adverse effects of glyphosate on the reproductive function of male rats and mice. A systematic and comprehensive literature search was performed using five different databases with different combinations of glyphosate exposure and sperm concentration. Eight studies were identified as qualified and a random effect model was conducted.

Materials and methods

Literature search strategy

A comprehensive literature search was performed on the association between glyphosate exposure on rodents and change of sperm concentration. The search was conducted in Pub Med, Web of Sciences, MEDLINE, TOXLINE, Embase, CNKI, and Wanfang databases from January 1990 up to November 2016, with a combination of the following keywords: glyphosate; round up; reproductive toxicity; testicular; testes; sperm reserves; sperm quality sperm concentrations; male; animal; rats; and mice. Further, titles and abstracts were examined of all papers obtained to identify other potential articles. The search and evaluation were conducted in November 2016.

Inclusion criteria

To identify qualified studies, title and abstract of each published paper were firstly screened followed by a full text screening if relevance was unclear. The following criteria were used to assess the paper:

a) Published in either English or Chinese between January 1990 and November 2016
b) Reported sperm concentrations
c) Reported results of analyses of an RCT
d) Glyphosate should be the only pesticide used in the experiment and cannot be combined with other pesticides or chemicals
e) Only *in vivo* experiment on rat and mouse models
f) In addition, publications with data available for further analysis were also included

Data extraction

Screening of eligible studies was conducted by two independent reviewers to reduce subjective bias and improve reliability. In addition, to mean value and corresponding SD of sperm concentration with or without glyphosate exposure, the following information was also extracted: first author, date of publication, experiment animal (rats or mice), strains of rats, strains of mice, animal age and body weight. All selected studies were controlled under standard animal experimental conditions. Some studies reported the exact age and bodyweight of the rodents before and after glyphosate exposure, others only provided a crude description, such as “mature” forage. According to the sexual cycle of rats, 21 day was regarded as weaned age and 80 day as mature age. Meanwhile, 54 day was considered as the average mature age for male mice. To analyse pooled data, two different approaches were used. First, a fixed effect model was constructed. Next, data from each study were fitted into a curve equation: changes in the amount of sperm as the dependent variable, and the dose effect of glyphosate on rodents as independent variable. Effective dose of glyphosate was multiplied by the body weight (mg), exposure frequency, duration of exposure (day), and exposure dose (mg/kg). A new value of an independent variable (dose effect of glyphosate) was recorded, to calculate the dependent variable (amount of sperm), which is the number of sperm by fitting equation. Finally, the fixed effect model was performed again.

Statistical analysis

STATA software 11.0 (STATA Corp, College Station, TX, USA) was used for all analyses. Heterogeneity was assessed by the I2 statistic and Q test with $P < 0.05$ and $I2 > 50\%$ indicating evidence of heterogeneity. Random effect model (Der Simonian Laird method) was used to calculate the pooled effect estimates in the presence or absence of heterogeneity. Sensitivity analysis was conducted by sequentially excluding each study to assess the stability of the results. Begg's test was performed to assess publication bias. All tests were two tailed and statistical significance was indicated by P values <0.05.

Results

Literature search

A total of 66 articles were identified of which 57 were excluded because the results were population based (21 articles), outcomes were irrelevant to reproduction (18 articles), and studies focused on the toxicity of glyphosate to spermatogonia (13 articles), rabbit studies (2 articles), not provided with mean value and standard deviation (3

articles). As stated by study author, eight papers on sperm concentration were selected for further review. Five out of these 8 studies revealed significantly reduced sperm concentration after glyphosate exposure. Several factors could interfere with study results:
(1) intraspecific variability,
(2) age at the start of experiments,
(3) duration of glyphosate exposure,
(4) body weight, and
(5) other potential biological variations.

Of the 8 selected studies, 5 used rats as animal models, and the other 3 used mice. Measurements of the included reports are displayed in Table 1.

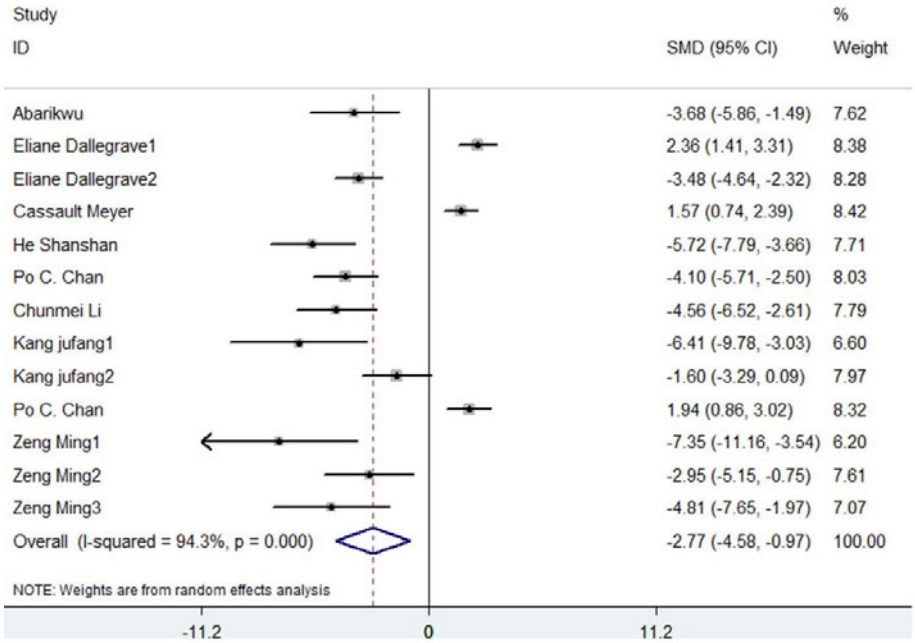
Table 1*: Characteristics of studies included in the meta-analysis of sperm concentration

Authors	Year	Rats/Mice	Strain	Unexposed Sperm concentrations (10^6/sperm/g testes)			Exposed Sperm concentrations (10^6/sperm/g testes)		
				n	mean	SD	n	mean	SD
Abarikwu	2015	rats	Wister	5	33.5	5	5	19.3	2.2
Eliane Dallegrave	2007	rats	Wister	15	44.2	4.2	15	57.4	6.7
Eliane Dallegrave				15	344.7	30.8	15	257.1*	17.9
Estelle Cassault-Meyer	2014	rats	SD	15	22	3	15	26	2
He Shenzhen	2016	rats	SD	10	8.89	0.98	10	3.72*	0.82
Po C. Chan	1992	rats	F344/N	10	610	36	10	486*	23
Chunmei Li	2016	rats	SD	8	40.72	2.905	8	29.42	1.956
Kang Jufang	2007	mice	KM	5	12.74	1.93	5	2.63	1.12
Kang Jufang				5	12.05	3.48	5	6.28*	3.84
Po C. Chan	1992	mice	B6C3F1	10	1162	44	10	1308	97
Zeng Ming	2010	mice	KM	5	12.54	1.8	5	2.64*	0.62
				5	12.68	2.26	3	6.91*	1.11
				5	12.35	1.57	4	6.27*	0.67

* Significant difference from unexposed, $P < 0.05$.

Fig. 1 shows the forest plot of the pooled estimate and 95% confidence interval of reduction in sperm concentrations after glyphosate exposure, calculated from a random effect model.

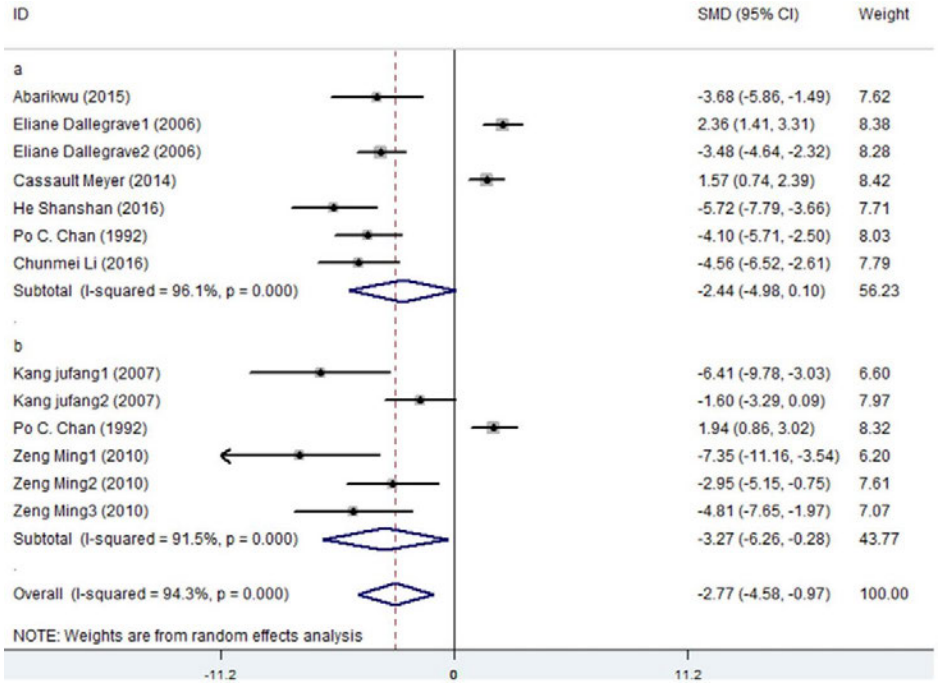
Fig. 1*: Forest plot of MDsperm with their 95% CIs performed by a random effect model



*Retrived from Cai, W., *et al*., Environmental Toxicology and Pharmacology 55 (2017) 148-155

Sperm concentration significantly decreased after exposure to glyphosate, and the pooled mean difference of sperm concentration (MDsperm) was 2.774 × 10^6/sperm/g/testis. Since there was significant heterogeneity between mice and rats, data were divided into two subgroups accordingly: mice and rats. As shown in Fig. 2, pooled MDsperm of rats was 2.436 × 10^6/sperm/g/testis, and pooled MDsperm of mice was 3.272 × 10^6/sperm/g/testis.

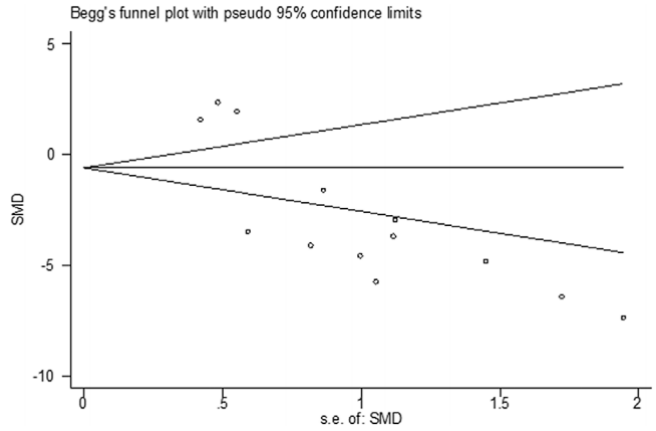
Fig. 2*: Forest plot of MDsperm with their 95% CIs performed by a random effect model. a = rat animal model; b = mice animal model



*Retrived from Cai, W., *et al.*, Environmental Toxicology and Pharmacology 55 (2017) 148-155

In the meta-analysis of paired testes weight, heterogeneity between the included 13 subgroups was significantly evident, thus a random effect model was used throughout the analysis and showed by the symmetric funnel plots (Fig. 3).

Fig. 3*: Funnel plot for MDsperm in the meta-analysis of sperm concentrations



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Publication bias was also observed in the meta-analysis of MDsperm. Begg's test showed an intercept of P = 0.200 for the meta-analysis of MDsperm, confirming the existence of publication bias.

Equation fitting

Measurements of the characteristics in the included papers were displayed in Table 2, with more independent experimental data from these studies. Weight was averaged across the data obtained in the papers. Because data utilization rate was not high in the meta-analysis and heterogeneity test results suggested a very large heterogeneity, each of the independent experimental data sets were used in order to fit the corresponding equations and improve the utilization rate of the data based on Table 2.

Table 2*: Specific characteristics of studies in the meta-analysis of sperm concentrations

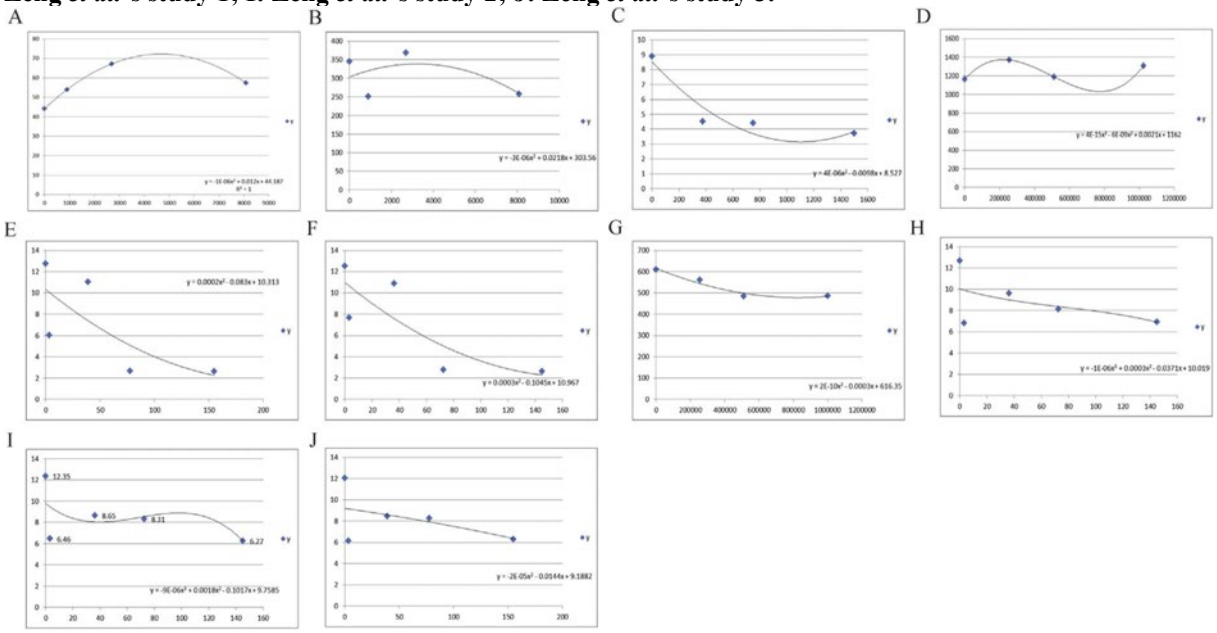
Authors	Year	Rats/mice	Strain	Body weight (kg)	Ages (day)	Duration of exposure (day)	The frequency of exposure	Exposure dose (mg/kg)	Total exposure dose(mg)	n	Unexposed Sperm concentrations and Exposed Sperm concentrations (10^6/ sperm/g testes)
Eliane Dallegrave	2006	rats	Wister	0.2995	65	0		0	0	15	44.2
						60	1[a]	50	898.5	15	53.9
								150	2695.5	15	67.2
								450	8086.5	15	57.4
Eliane Dallegrave[b]	2006	rats	Wister	0.2995	140	0		0	0	15	344.7
						140	1[a]	50	898.5	15	251
								150	2695.5	15	368.7
								450	8086.5	15	257.1
He Shanshan	2016	rats	SD	0.05	30	0	0	0	0	10	8.89
						30	1	250	375	10	4.52
								500	750	10	4.42
								1000	1500	10	3.72
PoC. Chan	1992	rats	F344/N	0.225	136	0	0	0	0		610
						91	1	12500	255937.5	10	561
								25000	511875	10	485
								50000	1023750	10	486
Kang Juan	2007	mice	KM	0.027		0	0	0	0	5	12.74
						5	3[c]	40	3.204	5	6.01
								290	38.715	5	11.04
								580	77.43	5	2.68
								1160	154.86	5	2.63
Kang Jufang	2007	mice	KM	0.027		0	0	0	0	5	12.05
							3	40	3.204	4	6.15
								290	38.715	5	8.45
								580	77.43	5	8.27
								1160	154.86	3	6.28
Po C. Chan	1992	mice	B6C3F1	0.023	143			0	0	10	
						284	1	12500	81650	10	1370
								25000	163300	10	1189
								50000	326600	10	1308
Zeng Ming	2010	mice	KM	0.023	56	0	0	0	0	5	12.54
						5	3	40	3	5	7.66
								290	36.25	5	10.89
								580	72.5	5	2.77
								1160	145	5	2.64
ZengMing[d]				0.023	84			0	0	5	12.86
							3	40	3	4	6.82
								290	36.25	5	9.63
								580	72.5	5	8.13
								1160	145	3	6.91
ZengMing[e]					91		3	0	0	5	12.35
								40	3	5	6.46
								290	36.25	5	8.65
								580	72.5	5	8.31
								1160	145	4	6.27

[a] The frequency of exposure is once a day.
[b] The data from second studies in the Eliane Dallegrave's article is measured in mice 140 days after exposured.
[c] The data from second studies in the Kang Jufang's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[d] The data from second studies in theZeng Ming's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[e] The data from second studies in theZeng Ming's article were measured after the mice were exposed to normal culture for a period of 5 weeks.

*Retrived from Cai, W., *et al*., Environmental Toxicology and Pharmacology 55 (2017) 148-155

Total glyphosate exposure dose was defined as the independent variable 'X' and the unexposed sperm concentrations and exposed sperm concentrations were defined as the dependent variable 'Y' in the fitted equation. Scatter plot and the fitted curve equation for each dataset were demonstrated in Fig. 4.

Fig. 4*: Fitted curve. A: Eliane *et al.*'s study 1; B: Eliane *et al.*'s study 2; C: He *et al.*'s study; D: Chan *et al.*'s study 1 (rat); E: Kang *et al.*'s study 1; F: Kang *et al.*'s study 2; G: Chan *et al.*'s study 2 (mice); H: Zeng *et al.*'s study 1; I: Zeng *et al.*'s study 2; J: Zeng *et al.*'s study 3.



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According to the exposure model, exposure time and the average body weight of adult rats and mice, the 'X' are re assigned 100, 600 and 900. Then 3 new Y values were shown in Table 3 after these adjustments. The negative values of the obtained Y should be of no practical importance.

Table 3*: Fitting equation and Y value corresponding to the X value

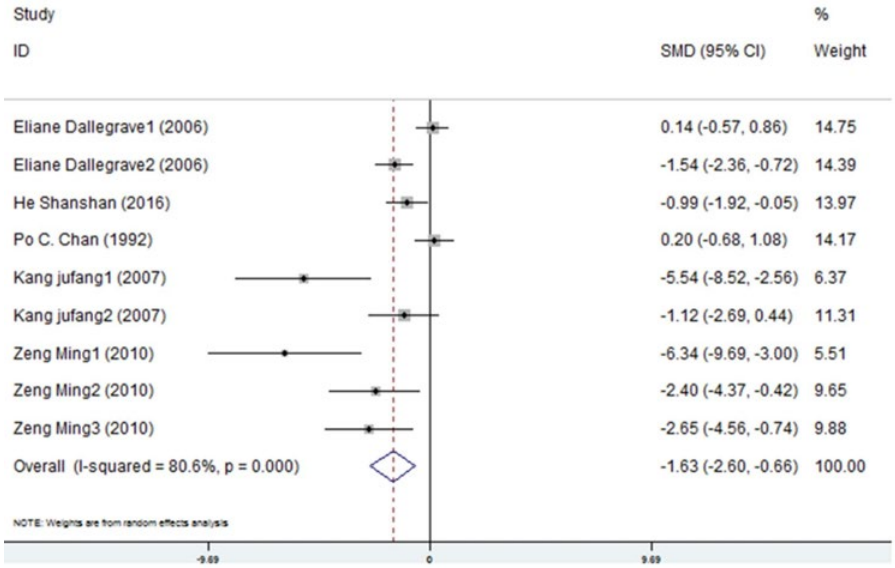
Authors	Strain	fitted equation	X_1 = 100 Y=	X_2 = 600 Y=	X_3 = 900 Y=
Eliane Dallegrave	Rats	$Y1 = -1E\text{-}06 \times^2 + 0.012x + 44.187$	45	51	54
Eliane Dallegrave[a]	Rats	$Y2 = -3E\text{-}06 \times^2 + 0.0218x + 303.56$	306	316	321
He shanshan	Rats	$Y3 = 4E\text{-}06 \times^2 - 0.0098x + 8.527$	8	4	3
PoC. chan	Rats	$Y4 = 2E\text{-}10 \times^2 - 0.0003x + 616.35$	616	616	616
Kang Iufang	Mice	$Y5 = 0.0002 \times^2 - 0.083x + 10.313$	4	33	98
Kang Jufang[b]	Mice	$Y6 = -2E\text{-}05 \times^2 - 0.0144x + 9.1882$	8	−7*	−20*
Po C. Chan	Mice	$Y7 = 4E\text{-}15 \times^3 - 6E\text{-}09 \times^2 + 0.0021x + 1162$	40001162	8640001163	29160001164
ZengMing	Mice	$Y8 = 0.0003 \times^2 - 0.1045x + 10.967$	4	56	160
ZengMing[c]	Mice	$Y9 = -1E\text{-}06 \times^3 + 0.0003 \times^2 - 0.0371x + 10.019$	8	−120*	−509*
ZengMing[d]	Mice	$Y10 = -9E\text{-}06 \times^3 + 0.0018 \times^2 - 0.1017x + 9.7585$	9	−1347*	−5185*

[a] The data from second studies in the Eliane Dallegrave's article is measured in mice 140 days after exposure.
[b] The data from second studies in the Kang Jufang's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[c] The data from second studies in the Zeng Ming's article were measured after the mice were exposed to normal culture for a period of 4 weeks.
[d] The data from second studies in the Zeng Ming's article were measured after the mice were exposed to normal culture for a period of 5 weeks.
* Significant difference from unexposed, $P < 0.05$.

*Retrived from Cai, W., *et al*., Environmental Toxicology and Pharmacology 55 (2017) 148-155

Finally, Y value was computed at X = 100 for meta-analysis again. Forest plot of the pooled estimate and 95% confidence interval for the reduction in sperm concentration in a random effect model was displayed in Fig. 5.

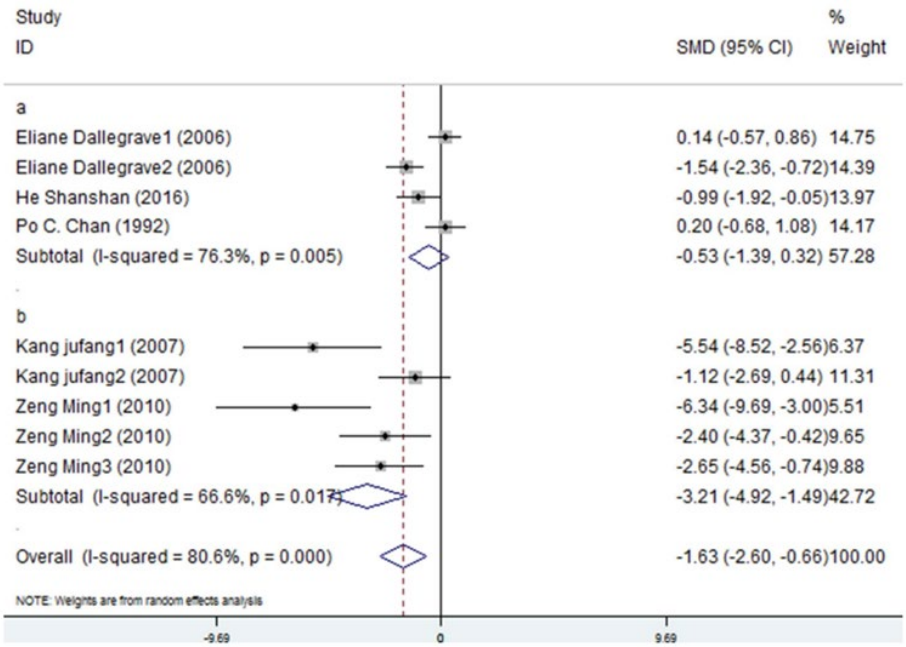
Fig. 5*: Forest plot of MDsperm of fitting equation with their 95% CIs performed by a random effect model



*Retrived from Cai, W., *et al*., Environmental Toxicology and Pharmacology 55 (2017) 148-155

Sperm concentration significantly decreased after exposure to glyphosate, and pooled MDsperm was 1.632 × 10^6/sperm/g/testis. Since there was significant heterogeneity between mice and rats, the data were further divided into two subgroups according to different animal models of mice and rats (a and b in Fig. 6), respectively. Pooled MDsperm of rats was 0.53 × 10^6/sperm/g/testis, and pooled MDsperm of mice was 3.21 × 10^6/sperm/g/testis.

Fig. 6*: Forest plot of MDsperm of fitting equation with their 95% CIs performed by a random effect model. a = rat animal model; b = mice animal model



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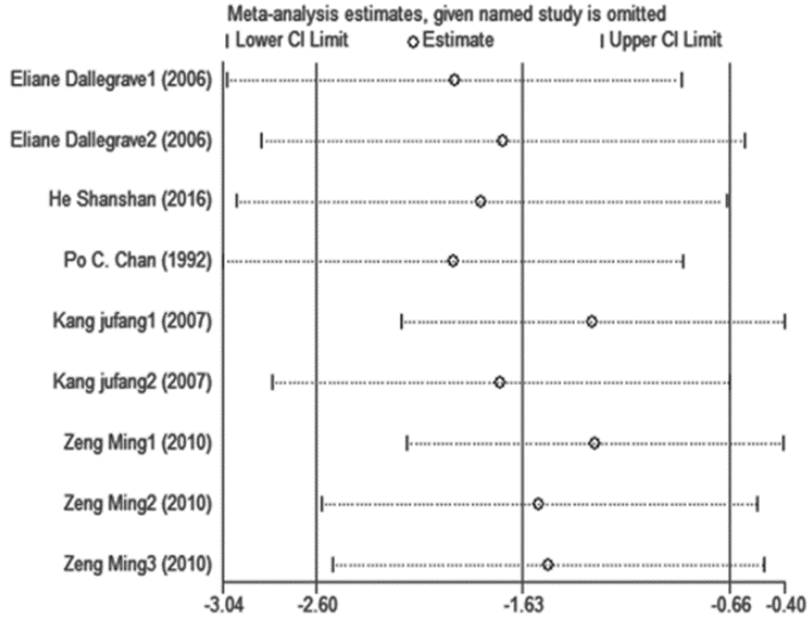
Heterogeneity among studies, as measured by the Q test and the I2 statistic, was lower than the first meta-analysis but remained significant in the random effect model (I2 = 80.6%). In mice group, I2 was 76.3%, while I2 was 66.6% in the rat group. There was significant heterogeneity in both groups in the random effect model. According to the sensitivity analysis, sequential removal of a single study did not result in notable changes, and this outcome suggested that the results were stable and robust (Fig. 7).

Fig. 7*: Sensitivity analysis



*Retrived from Cai, W., *et al*., Environmental Toxicology and Pharmacology 55 (2017) 148-155

Conclusion (study author)
In conclusion, results from our meta-analysis suggested that exposure to glyphosate caused a decrease in sperm concentration in rodents (both mice and rats), and consequently, impose an adverse effect on reproductive health. It is desirable to extend the study of glyphosate and its influence on reproductive health in humans and other mammals.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: Re-evaluation of pooled literature data.

Further points for clarification:
This study is a combination of a systematic literature review and a meta-analysis investigating if there is an association between glyphosate exposure and decreased sperm concentration in rats and mice. After the screening of the literature, 8 articles were graded relevant to perform a meta-analysis. The results suggest that glyphosate exposure decreases sperm concentration in rats and mice. Nevertheless, the authors only seemed to look at English and Chinese written articles. Out of the 8 selected studies, 5 showed reduced sperm count.

Assessment and conclusion by RMS:
This study is a combination of a literature review and a meta-analysis from 8 English and Chinese written articles between January 1990 and November 2016 in rats (5 studies) and mice (3 studies), as stated by study author. However, when looking at Table 1 it seems that 9 studies are included in the quantitative synthesis.

The results suggest that exposure to glyphosate caused a decrease in sperm concentration in both rats and mice. However, several factors may interfere with the results such as intraspecific variability, age of animals at start of experiments, dose and duration exposure, body weight and potential strain differences. Furthermore, the test substance used in the selected studies is not sufficiently characterized in this review report, and the selected studies are inadequately described. Also, the original articles are not available to confirm the results of these studies.

The reference is considered as supplementary data only (review article) and will not be given further significance in this report.

Category B – Owagboriaye, F.O. *et al.*

Data point	CA 5.6
Report author	Owagboriaye F. O. *et al.*
Report year	2017
Report title	Reproductive toxicity of Roundup herbicide exposure in male albino rat
Document source	Experimental and Toxicologic Pathology (2017), Vol. 69, No. 7, pp. 461-468
Guidelines followed in study	No guideline specified in report
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary

Aim of the study
The aim of the study was to assess the effect of Roundup on the reproductive capacity of adult male albino rats. Eight male albino rats/dose were administered daily via gavage with 3.6, 50.4 and 248.4 mg/kg bw of glyphosate in 0.25 ml/100 g for 12 weeks. The control group was administered 0.25 ml/100 g distilled water for 12 weeks. At the end of the study, blood sample, epididymal sperm cells and testicular tissue were collected from the rats to assess reproductive hormones (testosterone, luteinizing hormone [LH], follicle-stimulating hormone [FSH] and prolactin), oxidative stress indices, epididymal sperm morphology, sperm count and motility and testicular histopathology.

Materials and methods

Test material:	Roundup (360 g/L of glyphosate in the form of 441 g/L potassium salt) from Monsanto Europe S.A./N.V., Antwerp, Belgium, O611, F-1059 3379
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Distilled water
Test animals:	Male rat
Strain:	Albino rats
Age/weight on arrival:	Not reported/200 ± 20 g
Source:	Department of Zoology, Olabisi Onabanjo University Ago-Iwoye, Nigeria
Housing:	Single housed in individual wooden cages (65 cm x 35 cm x 50 cm)
Acclimatisation period:	7 days
Diet:	Rat chow, *ad libitum*
Water:	Not specified, *ad libitum*
Environmental conditions:	Temperature (25 ± 5°C) Relative humidity (65 ± 5%) Light/dark cycle 12 h/12 h (lights on at 08:00 h)

Animals and treatment
Food and water were available *ad libitum*.

Three groups of male rats (strain not specified) (8 males/group) were administered by oral gavage with Monsanto Roundup herbicide at concentrations of (3.6, 50.4 and 248.4 mg/kg bw/day of glyphosate) for 12 weeks, while rats in the control group were administered with distilled water.

Roundup was diluted in a watery suspension and administered daily via gavage in a volume of 0.25 mL/100 g/bw. The values are within the limits of NOAEL and equivalent to 1/1000, 1/100, and 1/20 of LD50 in rat.

At the end of the exposure period, blood sample, epididymal sperm cells and testicular tissue were collected from the rats in each group for laboratory analysis.

Sample collections
Blood samples were collected into clean sample tubes by retro-orbital sinus with micro haematocrit tube in baseline conditions between 8:00 a.m. and 10:00 a.m. The blood samples were centrifuged at 2500 g for 10 min and the obtained sera samples were stored in ice-cold until used for the hormonal assay. The rats were sacrificed by cervical dislocation and testes were removed by dissection and washed using saline solution.

Parts of this tissue were used to assay for antioxidant while the remaining parts were subjected to histopathological examination. Epididymis of the rats was dissected out and sperm cells were collected into 10 mL of 0.87% warmed normal saline for sperm abnormality assessment.

Hormonal assay
The sera obtained were analysed to determine the concentration of testosterone, FSH, prolactin, and LH using the enzyme-linked immunosorbent Assay (ELISA). The ELISA kits were obtained from Biocheck, USA.

Lipid peroxidation end product, malondialdehyde (MDA), was measured as thiobarbituric acid reactive substance (TBARS). The levels of reduced glutathione (GSH) were assayed. In addition, the activities of antioxidant enzymes were conducted according to standard procedures, as well as catalase (CAT) activity, superoxide dismutase (SOD) and glutathione peroxidase (GPx).

Epididymis sperm morphology assay and assessment
Sperm motility and count were carried out as well as sperm morphology assay. Epididymal sperm cells were suspended in normal saline and 1% Eosin Y stain. The smear was prepared and the slides were allowed to air-dried and coded for subsequent microscopic examination at 1000-fold magnification. One-thousand sperm cells were assessed for morphological abnormalities for each rat.

Histopathological examination of the testes
A portion of the testes excised from rats in each group was fixed in Bouin's fluid for 48 h and routinely processed for paraffin embedding. Sections of 4 μm thickness were taken serially with a Rotary Microtome and then, processed in alcohol-xylene series and were stained with hematoxylin and eosin (H & E). The prepared slides were examined at × 400 magnification.

Data analysis
Data obtained were presented as mean ± standard error of mean (SEM). Statistical analyses for all measurements were performed using Statistical Package for Social Sciences (SPSS) version 20.0. The mean, standard error of the mean, analysis of variance (ANOVA) and correlation coefficient were conducted. Post Hoc test was done using the Student-Newman-Keuls (SNK). $P < 0.05$ was considered to be statistically significant.

Results
Level of reproductive hormones in the blood
Testosterone, FSH and LH levels were statistically significantly decreased in the Roundup exposed rats compared to the control. The level of prolactin hormone in the blood of the rats was an inverse of testosterone, FSH and LH. Prolactin was observed to be significantly higher in Roundup exposed rats. The observed increase or decrease in the hormonal level appeared to be dose-dependent (Table 1).

Table 1*: Level of the reproductive hormone in the blood of the rats exposed to Roundup at different concentrations of glyphosate

Treatments	Testosterone (ng/ml)	FSH (mIu/ml)	LH (mIu/ml)	Prolactin (ng/ml)
Control	3.17 ± 0.12^{a}	9.49 ± 0.26^{a}	8.29 ± 0.34^{a}	2.36 ± 0.21^{d}
3.6 mg/kg bw	2.75 ± 0.09^{b}	7.84 ± 0.26^{b}	7.11 ± 0.09^{b}	3.09 ± 0.11^{c}
50.4 mg/kg bw	2.10 ± 0.07^{c}	6.11 ± 0.33^{c}	5.42 ± 0.35^{c}	4.05 ± 0.19^{b}
248.4 mg/kg bw	1.07 ± 0.07^{d}	3.19 ± 0.12^{d}	2.18 ± 0.12^{d}	6.54 ± 0.35^{a}

^{abcd}Mean (± Standard deviation) in the same column having similar superscript are not significantly different at $P < 0.05$.
*Retrived from Owagboriaye, F.O., *et al*., Environmental Toxicology and Pharmacology 69 (2017) 461-468

Of great interest is the high level of serum prolactin (hyperprolactinemia) observed in the rats exposed to Roundup in this study. Other authors previously submitted that hyperprolactinemia impairs male gonadal

functions by acting at various levels, possibly by decreasing gonadotropin-releasing hormone (GnRH) pulse generator activity and might be inhibiting testosterone secretion at the level of Leydig cells as well. However, a high level of prolactin observed in the rats exposed to Roundup could also be responsible for the corresponding decrease in their serum testosterone level.

Level of antioxidant parameters in the testes tissue

Mean levels of SOD, GSH, CAT, and GPx in the testes of the Roundup exposed rats were significantly reduced compared to the control rats, and the reduction was observed to be dose-dependent. However, the mean level of MDA in the testes was statistically significant from the control rats and rose with increasing concentrations of the glyphosate exposure (Table 2).

Table 2*: Level of oxidative stress markers in the testes of rats exposed to Roundup at varying concentrations of glyphosate

Treatments	SOD (U/mg protein)	GSH (U/g tissue)	CAT (U/mg protein)	GPx (U/mg protein)	MDA (nmol/g tissue)
Control	6.88 ± 0.26[a]	11.96 ± 0.16[a]	10.39 ± 0.41[a]	17.51 ± 0.42[a]	21.97 ± 0.41[d]
3.6 mg/kg bw	5.07 ± 0.03[b]	9.04 ± 0.11[b]	8.93 ± 0.18[b]	14.11 ± 0.30[b]	27.92 ± 0.18[c]
50.4 mg/kg bw	3.65 ± 0.15[c]	5.15 ± 0.06[c]	6.21 ± 0.16[c]	9.27 ± 0.28[c]	38.90 ± 0.23[b]
248.4 mg/kg bw	0.93 ± 0.46[d]	2.37 ± 0.14[d]	3.40 ± 0.21[d]	5.10 ± 0.51[d]	49.02 ± 0.32[a]

[abcd]Mean (± Standard deviation) in the same column having similar superscript are not significantly different at $P < 0.05$.

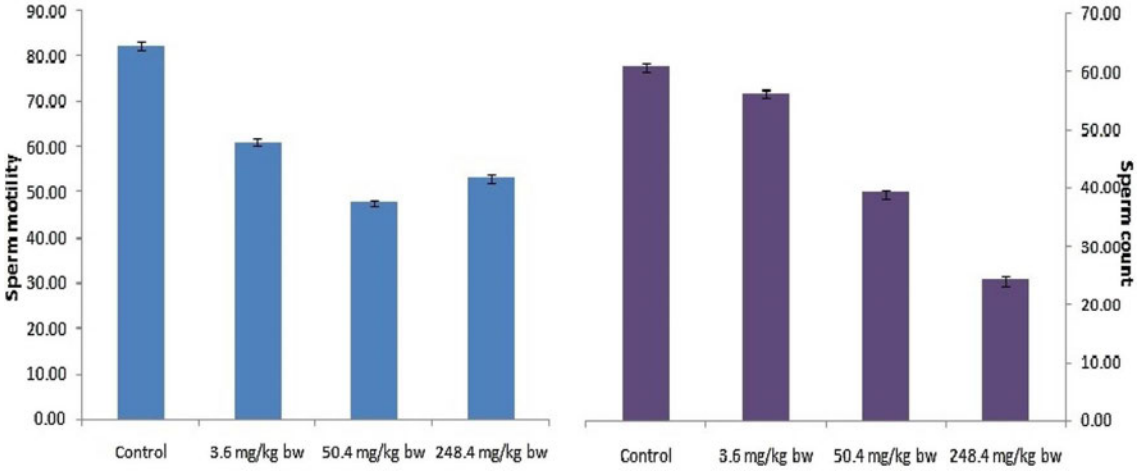
*Retrived from Owagboriaye, F.O., *et al*., Environmental Toxicology and Pharmacology 69 (2017) 461-468

Increased level of MDA and reduced activities of the antioxidant enzymes in the testes of the exposed rats may suggest the induction of oxidative stress by the Roundup. It is worthy to note that glyphosate would have induced production of ROS in the testes of the exposed rats, consequently induced the peroxidation of polyunsaturated fatty acids in the membrane of the testes, which led to the formation of MDA, one of the by-products of lipid peroxidation as previously observed. Reductions in the activities of GSH, SOD and CAT in the testes of the exposed rats may be attributed to probably the excessive production of free radicals or ROS the glyphosate may have inflicted on the testes after the exposure. However, it is reasonable to relate the presence of lipid peroxidation and reduced GSH observed in the testes of the exposed rats in this study to the reduction of their SOD, CAT and GPx activities that in turn, can be adduced to the oxidative stress inflicted on the testes tissue by Roundup.

Sperm motility and count

The percentage of sperm motility and sperm count observed in the rats exposed to Roundup at varying concentrations of glyphosate were statistically significant compared to the control (Fig. 1). The lowest sperm count was observed in the rats exposed to Roundup at 248.4 mg/kg bw of glyphosate. However, the lowest percentage of sperm motility was observed in the rats exposed to Roundup at 50.4 mg/kg bw of glyphosate.

Fig. 1*: Sperm motility (%) and sperm count (×10^6 cells/mL) in rats exposed to Roundup at different concentrations of glyphosate.



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Reductions in epididymal sperm count and percentage sperm motility observed in rats exposed to Roundup may be attributed to the induction of ROS in the testes and epididymis of the exposed rats. Hyperprolactinemia in

men has been shown to typically result in hypogonadism which led to decreased libido, erectile dysfunction and abnormal semen quality (Corsello et.al. 2003)*. In a similar trend, prolactin receptors have been demonstrated in the spermatogonia, seminiferous epithelium, and spermatocytes, suggesting, that prolactin might function in normal spermatogenesis. Hence, low sperm count and motility observed in this study among the exposed rats to Roundup may also be attributed to the increased in their serum prolactin.

* Corsello, S.M., Ubertini, G., Altomare, M., Lovicu, R.M., Migneco, M.G. and Rota, C.A., 2003. Giant prolactinomas in men: effect of cabergoline treatment. Clin. Endocrinal. 58, 662-670.

Sperm morphology assay

Total abnormal sperm cells in rats exposed to Roundup at 3.6, 50.4 and 248.4 mg/kg bw of glyphosate respectively were observed. These values are fractions of the 1000 sperm cells assessed and were statistically significant compared to the control. Abnormal sperm cells with a bent tail, without a head, damaged head, without a hook, banana shape, and without tail were observed in the rats exposed to Roundup and these values were statistically significant with increasing concentrations of glyphosate (Table 3).

Table 3*: Sperm morphological abnormality count in rats exposed to Roundup at different concentrations of glyphosate

Concentration	Bent tail	Without Head	Damaged Head	Without Hook	Banana Shape	Without Tail	Total Abnormal	Percentage Abnormalities
Control	11.50 ± 1.29^{d}	0.00 ± 0.00^{d}	1.25 ± 1.26^{d}	1.00 ± 0.82^{d}	5.75 ± 0.96^{d}	0.00 ± 0.00^{c}	19.50 ± 2.52^{d}	1.95 ± 0.25^{d}
3.6 mg/kg bw	28.75 ± 0.96^{c}	11.25 ± 1.26^{c}	21.75 ± 1.71^{c}	8.75 ± 0.96^{c}	30.25 ± 1.26^{c}	10.25 ± 1.26^{b}	111.00 ± 4.76^{c}	11.10 ± 0.48^{c}
50.4 mg/kg bw	37.25 ± 1.26^{a}	17.75 ± 1.26^{b}	36.75 ± 1.89^{b}	20.50 ± 1.29^{b}	58.50 ± 2.65^{a}	18.00 ± 3.56^{a}	188.75 ± 4.11^{b}	18.88 ± 0.41^{b}
248.4 mg/kg bw	32.00 ± 0.82^{b}	35.75 ± 1.26^{a}	59.50 ± 2.89^{a}	48.00 ± 2.58^{a}	42.25 ± 1.26^{b}	21.00 ± 3.37^{a}	238.50 ± 1.73^{a}	23.85 ± 0.17^{a}

^{abcd}Mean (± Standard deviation) in the same column having similar superscript are not significantly different at $P < 0.05$. Mean (± Standard deviation) are fractions of the 1000 sperm cells assessed

*Retrived from Owagboriaye, F.O., *et al*., Environmental Toxicology and Pharmacology 69 (2017) 461-468

Induction of abnormal sperm cells could be a result of an abnormal chromosome (Bruce et.al., 1974)*, minor alterations in testicular DNA (Giri et.al., 2002)* or point mutation (Narayana et.al., 2002)*. The occurrence of sperm head abnormalities has been attributed, to the chromosomal aberrations that occur during the packaging of genetic material in the sperm head or occurrence of a point mutation in testicular DNA (Bruce and Heddle, 1979). Thus, glyphosate interactions with the genetic material in the testes of the exposed rats during spermatogenesis, which can probably lead to the observed increase in their abnormal sperm cells are reasonably suggested.

*Bruce, W.R., Furrer, R., Wyrobek, A.J., 1974. Abnormalities in the shape of murine sperm after acute testicular x-irradiation. Mutat. Res. 23, 381-386

*Giri, S., Prasad, S.B., Giri, A. and Sharma, G.D., 2002. Genetoxic effect of malathion: an organophosphorus insecticide, using three mammalian bioassays *in vivo*. Mutat. Res. 514, 223-231.

*Narayana, K., D´Souza, U.J. and Seetharama Rao, K.P., 2002. Ribavirin-induced sperm shape abnormalities in Wistar rat. Mutat. Res. 513, 193-196.

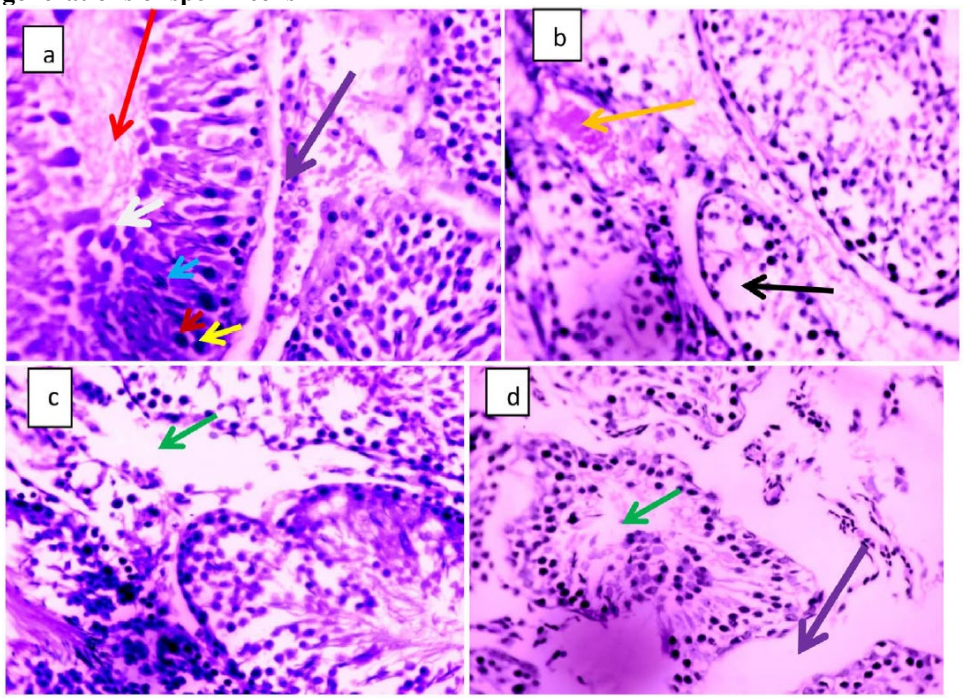
*Bruce, W. and Heddle, J., 1979. The mutagenicity of 61 agents as determined by micronucleus, Salmonella and sperm abnormality assays. Can. J. Cytol Genet. 21, 319-334.

Histopathological assessment of the testes

Control group revealed the normal cellular architectural structure of the tissues while progressive degenerative lesions were observed in rats testes exposed to different concentrations of glyphosate (Fig. 2). Degeneration of testicular tissue, tubular hyalinization, a moderately depleted amount in the generation of sperm cells and interstitial cells were observed in the testes of the rats exposed to Roundup at 3.6 mg/kg bw of glyphosate. Severe testicular distortions characterized by vacuolation of the seminiferous tubule, alterations in the generation of sperm cells and reductions of interstitial cells in rats exposed to Roundup at 50.4 mg/kg bw of glyphosate were observed. Rats exposed to Roundup at 248.4 mg/kg bw of glyphosate showed marked vacuolation of the seminiferous tubule, loss of interstitial cells with marked reductions in sperm cells generation.

Fig. 2*: Testes tissue (H & E stain; ×400) of (a) control showing normal seminiferous tubule (red arrow), interstitial cells (purple arrow), spermatogonia (yellow arrow), primary spermatocytes (dark red arrow), secondary spermatocytes (blue arrow) and spermatids (white arrow), (b) rats exposed to Roundup at 3.6 mg/kg bw of glyphosate showing degeneration of testicular tissue (black arrow) and tubular hyalinization (orange arrow) with a moderately depleted amount in all the stages of sperm cells, (c) rats exposed to Roundup at 50.4 mg/kg bw of glyphosate showing marked vacuolation of the seminiferous tubule (green

arrow) affecting all the generations of sperm cells and moderate reduction of and scattered interstitial cells and (d) rats exposed to Roundup at 248.4 mg/kg bw of glyphosate with marked testicular lesions; vacuolation of the seminiferous tubule, loss of interstitial cells with marked reductions in all the generations of sperm cells



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Degenerative changes in the seminiferous tubule caused by a chemical agent may indicate the interference of the chemical with the spermatogenesis process. Thus, glyphosate may have directly interfered with the process of spermatogenesis in the testes of the exposed rats. This could probably be a pre-requisite for the observed abnormal sperm morphology produced by the damaged testes.

Relationship of reproductive hormones in the blood and the testicular antioxidant defence system with percentage sperm motility, sperm count and total sperm abnormality count.

The relationship between blood levels of reproductive hormones (except prolactin) and testes antioxidant (except MDA) with sperm motility and sperm count were positive and significantly strong (Table 4). However, the blood level of prolactin and testes level of MDA were negatively correlated with sperm motility and sperm count. On the other hand, the relationship between blood levels of reproductive hormones (except prolactin) and testes antioxidant (except MDA) with total sperm abnormality count were strong and significantly negative.

Table 4*: Relationship of reproductive hormones in the blood and the testicular antioxidant defence system with percentage sperm motility, sperm count and total sperm abnormality count

		Sperm Motility	Sperm count	Total sperm abnormality
Blood	Testosterone	0.654**	0.841**	−0.801**
	FSH	0.738**	0.865**	−0.857**
	LH	0.668**	0.815**	−0.803**
	Prolactin	−0.638**	−0.844**	0.806**
Testes	SOD	0.749**	0.847**	−0.857**
	GSH	0.762**	0.892**	−0.889**
	CAT	0.701**	0.884**	−0.858**
	GPx	0.737**	0.899**	−0.878**
	MDA	−0.708**	−0.889**	0.861**

* Correlation is significant at P < 0.01 (Pearson correlation).

*Retrived from Owagboriaye, F.O., *et al*., Environmental Toxicology and Pharmacology 69 (2017) 461-468

Conclusion (study author)

The conducted study has shown that Roundup has the capacity to induce reproductive toxicity in the male reproductive system of the exposed animal. It is also a potent endocrine disruptor. We can conclude that the

disruption in the normal testicular cellular architecture observed in the rats exposed to Roundup, in this study, which could, probably, led to abnormal hormonal secretion and abnormal sperm properties, may be attributed to the oxidative stress inflicted on the gonad of the exposed rats by the active ingredient in Roundup.

Comment by applicant:
Nevertheless, it should be kept in mind, that commercial formulation of Roundup is a mixture of glyphosate (active ingredient) with varying and unspecified surfactants and that the mechanisms of toxicity of glyphosate formulations are complicated. The observed toxicity in this study may not directly result from the glyphosate composition but by the herbicide's complex and variable mixtures.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: Formulation tested *in vivo* (Roundup 441 g/L potassium salt, 360 g/L a.e.).

The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains potassium salt, thus the composition differs to the EU representative formulation MON 52276. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Only 8 rats/dose were tested, versus the required 10 rats/sex/dose under OECD 408. In the absence of historical control data from the lab, the results presented across many parameters with low standard deviations, are difficult to verify.

No batch, purity, or CAS No. of the test substance reported. Strain of test animals not specified. Diet of animals not mentioned. No positive control. No historical control data given.

Assessment and conclusion by RMS:
In this study groups of 8 male albino rats (strain of animals not specified) received the formulation Roundup (360 g/l of glyphosate in the form of 441 g/l potassium salt) by gavage at doses of 3.6, 50.4 and 248.4 mg/kg bw/day in 0.25 ml/100 g bw for 12 weeks. Male albino rats in the control group received treatment with distilled water. At the end of the study, blood sample, epididymal sperm cells and testicular tissue were collected from the rats to assess reproductive hormones, oxidative stress indices, epididymal sperm morphology, sperm count and motility and testicular histopathology.

The reproductive hormones testosterone, FSH and LH levels in blood appeared to be dose dependently decreased while the prolactin levels appeared to be dose dependently increased. Oxidative stress markers in testes were observed to be dose dependently reduced compared to control rats with exception of MDA which increased with increased doses of Roundup. Sperm motility and count were also decreased in rats exposed to Roundup. Histopathology assessment of the testes from the control group revealed normal cellular architectural structure while progressive degenerative lesions were observed in rat testes exposed to Roundup.

The study is considered as supplementary data only. The study is carried out with a formulation of glyphosate, thus effects caused by co-formulants cannot be excluded. The study is reliable with restrictions because of the following reasons: the test substance is not sufficiently characterised (particularly, purity and batch not specified), small group sizes of animals used, strain of animals not specified, no details on food, clinical signs and body weight not specified, no historical control data and positive control missing.

Category B – Milesi, M.M. *et al.*

Data point	CA 5.6
Report author	Milesi, M. M. *et al*.
Report year	2018
Report title	Perinatal exposure to a glyphosate based herbicide impairs female

	reproductive outcomes and induces second generation adverse effects in Wistar rats
Document source	Archives of Toxicology (2018), Vol. 92, No. 8, pp. 2629 2643
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary

Aim of the study
The aim of the study was to investigate whether perinatal exposure of 2 and 200 mg/kg bw/day of a glyphosate-based herbicide (GBH) alters female reproductive performance, and/or induces second-generation effects related to congenital anomalies or growth alterations. Pregnant rats (F0) were administered GBH through food in a dose of 2 mg or 200 mg of glyphosate/kg bw/day from gestational day 9 until weaning. The serum concentrations of glyphosate and AMPA were determined at the end of the lactation period. Body weight gain and vaginal canal opening of F1 females were recorded. Sexually mature F1 females were mated and their reproductive performance assessed by determination of pregnancy rate and on gestational day 19, the number of corpora lutea, the implantation sites and resorption sites. To evaluate possible second generation effects on F2 offspring, foetal morphology on gestational day19, foetal length and weight, and the placental weight were analysed.

Materials and methods

Test material:	Glyphosate formulation MAGNUM SUPER II (66.2 % glyphosate potassium salt (equivalent to 54 % w/v glyphosate acid), Grupo Agros S.R.L., Argentina
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Rat
Test animals:	Wistar (inbred strain)
Strain:	Not reported
Age/weight on arrival:	Department of Human Physiology (Universidad Nacional del Litoral)
Source:	Individually in stainless steel cages with wood bedding
Housing:	Not reported
Acclimatisation period:	Laboratory pellet chow based paste (Nutrición Animal, Santa Fe, Argentina)
Diet:	Tap water, *ad libitum*
Water:	Temperature: 22± 2°C
Environmental conditions:	Light/dark cycle: 14 hr light 10 hr dark (lights on from 06:00 to 20:00)

Experimental design
The experimental design is visualised in Fig. 1. Nulliparous female rats at the proestrus stage were caged overnight with males of proven fertility. Every morning, vaginal smears were performed to check for the presence of spermatozoa. The first day on which a sperm positive smear was detected was considered gestational day 1 (GD1). Seven pregnant females (F0) per group were administered the test substance, a GBH mixed with laboratory pellet chow based paste at 2 or 200 mg of glyphosate/kg bw/day starting on gestational day (GD) 9 until the end of weaning (lactational day (LD) 21). Additionally, a control group consisting of seven pregnant females treated with the same diet but without glyphosate was included.

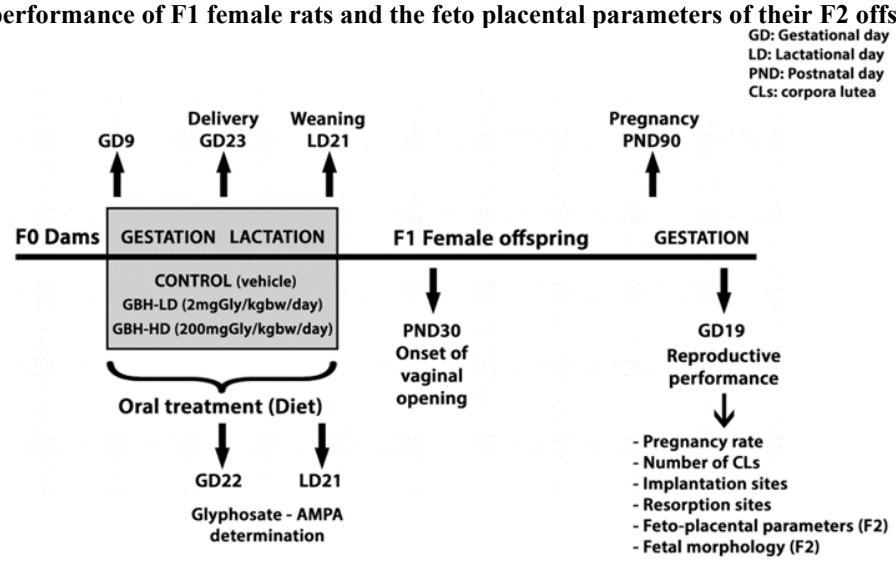
The laboratory chow based paste was prepared by blending optimized quantities of pellet chow (Nutrición Animal, Santa Fe, Argentina) and water; for GBH treatment groups a glyphosate commercial formulation was added to the water according to the above described doses. The mixture was covered and stood overnight, after that it was homogenized to form a paste and chow balls were prepared for each treatment. The pellet based paste for control and GBH groups was prepared freshly, i.e., the same day the food was replaced.

Glyphosate integrity in the dietary matrix was checked by measuring its concentration during three consecutive days by ultra performance liquid chromatography tandem mass spectrometer (UHPLC MS/MS). A preliminary study was carried out to ensure that the addition of GBH does not alter food consumption, and to estimate the

average amount of chow based paste daily consumed by F0 dams during pregnancy and lactation. From these, the amount of the active ingredient (glyphosate) was calculated to be added to the pellet chow.

To determine the dose of glyphosate administered, individual maternal weight and food intake were recorded three times per week throughout the treatment period. With these data, the relative body weight gain and loss of F0 dams were calculated during gestation and lactation periods, respectively. After delivery (postnatal day (PND) 0), F1 pups were weighed and sexed according to the anogenital distance, and litters of eight pups (preferably four males and four females) were left with F0 lactating mothers. The following parameters were analysed: length of gestation, birth weight of male and female pups, litter size and maternal care. At weaning (PND21), female offspring were housed in groups of four rats according to the treatment group (control or GBH exposed) with free access to pellet laboratory chow and tap water. Body weight of F1 females was recorded between birth and PND90 (PND 1, 7, 14, 21, 60 and 90). The onset of puberty was determined by checking the vaginal canal opening on PND30.

Fig. 1*: Schematic representation of the experimental protocol used to study the effects of perinatal (gestation plus lactation) exposure to low doses of a GBH (GBH LD and GBH HD) on the reproductive performance of F1 female rats and the feto placental parameters of their F2 offspring



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Glyphosate and AMPA determination in F0 dams' serum

Trunk blood of F0 mothers was collected at two different time points: (1) at the end of gestation, GD22, and (2) at weaning, LD21. The concentrations of glyphosate and its metabolite AMPA were measured. The analytical procedure consisted of FMOC derivatization followed by analysis using UHPLC MS/MS. Glyphosate (97.0%), AMPA (98.0%), glyphosate FMOC (91.5%), AMPA FMOC (98.0%) and isotopically labelled standards (ILS) [1,2 13C2 15N] glyphosate (98%) and [13C2 15N] AMPA were from Dr. Ehrenstorfer (Augsburg, Germany). All other supplies used were of the best commercially available analytical grade. Five hundred µL of rat serum (blank, blank spiked with standards, or experimental serum samples) were transferred to an Eppendorf tube, and 250 µL of acetonitrile (MeCN) was added. The mixture was vortexed for 1 min, placed in an ultrasound device for 10 min, and centrifuged at 15,000 rpm for 10 min at room temperature to precipitate the proteins. Five hundred µL of the supernatant were collected and transferred to a different Eppendorf tube and the same precipitation step was repeated once again. The supernatant (500 µL) was then spiked with internal standard (40 µL of ILS 1 mg/L) and was derivatized with the addition of 84 µL borate buffer (40 mM pH 9), 84 µL FMOC Cl (6 g/L), and acetonitrile. After a 2 h reaction at room temperature, the derivatization was quenched by acidification to pH 3 with formic acid. After the derivatization reaction was completed, the extracts were cleaned up by liquid liquid partition with dichloromethane (500 µL extract/500 µL DCM). Finally, 10 µL of this solution was injected into the UHPLC MS/MS system. Validation was carried out following the Document SANTE/11945 (SANTE 2015), by determining recovery, selectivity, limits of quantification and detection, linearity, precision and accuracy. The recovery of glyphosate and AMPA was determined in 6 replicates at 3 concentrations levels (1, 10 and 100 µg/L), comparing the peak areas of glyphosate and AMPA from standard samples with those from: (1) extracted blank serum samples from control rats, spiked with the same amounts of the compounds and then treated as described above (Recovery assay); (2) and to check the possible matrix effect

on the ESI ionization, extracted blank serum samples from control rats that were treated as described above and then spiked with the same amounts of glyphosate and AMPA (matrix matched serum). The average percentage of recoveries were between 80-100% with relative standard deviation (RSD) lower than 20% for all residues. To check the selectivity of the method, extracts from blank and spiked serum samples were assayed. The limit of detection (LOD) and quantification (LOQ) were determined by injecting a number of extracts from blank serum samples (n = 6) and measuring the magnitude of the background response. The LOD was experimentally estimated as three times the signal to noise ratio (S/N) and LOQ as the lowest concentration that could be measured with an intra assay precision CV% and relative bias less than 20%. The LOD and LOQ for glyphosate and AMPA were 1 and 2 µg/L, respectively. Precision was expressed as the % RSD and accuracy was calculated through the relative error (% RE).

Evaluation of reproductive performance in F1 females
On PND90, F1 females (control n=25, GBH-LD exposed n=20 and GBH-HD n=20) were caged with untreated fertile males. The presence of spermatozoa in vaginal smears was registered as an index of pregnancy. The pregnancy rate was calculated as the number of pregnant females/number of females housed with a male × 100. Pregnant females with sperm positive smears were housed separately and submitted to a fertility test on GD19. The ovaries from the pregnant rats were dissected, and the number of profusely irrigated CLs was counted by direct visualization using a stereomicroscope (Leica Corp., Buffalo, NY, USA). The two horned uteri were removed and visually inspected to identify the number of RS and IS. The RS were defined as endometrial sites with an appended amorphous mass without a foetus. The number of IS was defined as the result of the total number of placentas with foetuses plus the total number of RS. With these data, the rate of pre implantation was calculated loss as follows: [number of CLs number of IS/number of CLs] × 100.

Feto placental parameters in F2 offspring
To determine second generation effects on foetal development, F2 foetuses were removed from uteri on GD19. Foetuses and their respective placentas were examined for their morphology and weighed; with these data, the placental index was calculated as follows: (placental weight/foetal body weight). Foetal length from the top of the head to the bottom of the buttocks (crown rump length) was also measured. F2 foetuses were classified according to their weight on GD19 as small (SGA) (< 10th percentile), appropriate (AGA) (10 90th percentile) or large (LGA) (> 90th percentile) for gestational age, using a frequency distribution curve. This curve was constructed with weight values of F2 foetuses from the control group of our colony (data not shown), which followed the classic bell shaped Gaussian distribution.

Statistical analysis
Results are expressed as the mean ± SEM. The data from the number of IS, CLs, and feto placental parameters were analysed using one way ANOVA followed by Dunnett's test for the multiple comparisons (after Bartlett's test for the homogeneity of the variance). The analysis of the number of RS was conducted using a generalized linear model with a negative binomial response with the glm.nb function from R statistical software. Occurrence of unilateral pregnancy was assessed using Fisher's exact test. Incidence of SGA or LGA F2 foetuses and the relative risk associated with these categories were analysed by Chi square test. Occurrence of congenital anomalies in F2 foetuses was also analysed by Chi square test. All statistical analyses were performed using R statistical software (The R Foundation for Statistical Computing version 3.4.1). Differences were considered significant at $p < 0.05$.

Results

Glyphosate concentration in the dietary mix
For the low (GBH LD) and high dose (GBH HD) diet mixture with glyphosate, the concentrations were within the expected values and did not change over the test period, suggesting glyphosate integrity was preserved (see Table 1). Glyphosate was not detected in the control diet.

Table 1*: Glyphosate concentrations in the dietary matrix along three consecutive days (period of food replacement).

	Day 1	Day 2	Day 3
Control	ND	ND	ND
GBH-LD	21 ± 1	22 ± 1	20 ± 1
GBH-HD	2688 ± 77	2699 ± 103	2540 ± 53

Data are shown as mean ± SEM of three independent determinations calculated on dry weight basis and expressed in mg kg^{-1}

ND not detected

*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

Data about F0 dams and glyphosate and AMPA serum levels

GBH treatment through food did not produce signs of embryotoxicity, abnormal maternal or nursing behaviours. None of the reproductive parameters was affected including, gestation length, number of live born pups per litter and the litter sex ratio. Further, weight at birth in GBH exposed F1 males or females was unaffected by treatment and no gross malformations were observed in F1 pups at birth. Moreover, no changes were detected in the F0 dams´ body weight gain during pregnancy. However, the relative body weight loss from LD1 until weaning was higher in F0 dams treated with the low dose of GBH (see Table 2).

Table 2*: Animal weight and characteristics of dams (F0) and their offspring in control and GBH exposed groups.

Features	Control	GBH-LD	GBH-HD
Body weight of dams (g) (GD0)	243.1 ± 3.7	246.4 ± 2.6	249.2 ± 4.8
Relative weight gain (%)[a]	33.8 ± 1.9	34.4 ± 3.3	26.3 ± 1.7
Relative weight loss (%)[b]	7.8 ± 2.3	18.3 ± 1.2*	9.4 ± 2.2
Length of gestation (days)	23	23	23
Litter size			
Female	5.0 ± 0.9	5.2 ± 0.7	4.8 ± 0.8
Male	4.9 ± 0.4	5.7 ± 0.5	4.4 ± 0.9
Total	9.9 ± 0.9	10.8 ± 0.6	9.2 ± 0.5
Birth weight (g)			
Female	5.57 ± 0.12	5.41 ± 0.11	5.42 ± 0.14
Male	5.89 ± 0.09	5.59 ± 0.09	5.87 ± 0.13
Total	5.73 ± 0.08	5.50 ± 0.08	5.65 ± 0.12

Data are shown as mean ± SEM

*Indicates a significant difference ($p < 0.05$) between the GBH-LD and the control group

[a]Relative weight gain = relative weight on the last day of pregnancy (GD23) minus relative weight on the GD9 of pregnancy (weight of GD9 of pregnancy = 100%)

[b]Relative weight loss = relative weight on the last day of lactation (LD21) minus relative weight on the first day of lactation (LD1) (weight of LD1 of lactation = 100%)

*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

Food intake during pregnancy and lactation was not affected in F0 dams exposed to the herbicide. Moreover, no changes were detected in the average body weight of F0 dams. The real average doses of glyphosate, calculated based on the dams average body weight and food consumption during pregnancy and lactation are shown in Table 3.

Table 3*: F0 daily GBH doses and serum glyphosate and AMPA levels.

	Control	GBH-LD	GBH-HD
Theoretical dose (mg/kg bw/day)	0	2	200
Food intake (g/day)	29.14 ± 0.61	29.39 ± 0.69	29.65 ± 0.42
Average body weight (g)	259.00 ± 3.48	257.90 ± 3.10	252.70 ± 4.60
Real average dose (mg/kg bw/day)	0	3.69 ± 0.07	352.20 ± 4.78
Serum glyphosate level (mg/L)	ND	0.039 ± 0.006	3.8 ± 1.2
Serum AMPA level (mg/L)	ND	ND	ND

Data are shown as mean ± SEM of 7 F0 dams/group

ND not detected

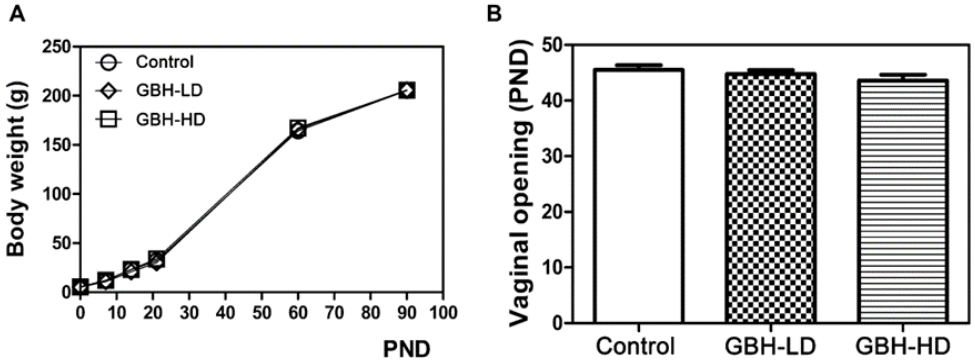
*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

Although the real glyphosate doses effectively reached in both GBH groups resulted in a bit higher than the theoretical doses (3.69 ± 0.07 instead of 2 mg/kg bw/day in GBH LD group, and 352.2 ± 4.78 instead of 200 mg/kg bw/day in GBH HD group), they were in the same order of magnitude. Moreover, as originally set out, a 100-fold difference was maintained between the two real glyphosate doses. The mean serum concentrations of glyphosate in F0 dams at the end of the lactation period (LD21) were: 0.039 ± 0.006 mg/L for GBH LD group, and 3.8± 1.2 mg/L for GBH HD group. Again, it is worth noting that a 100-fold difference was observed when comparing glyphosate concentrations of F0 dams from the two treated groups. As for AMPA serum concentrations, the main metabolite of glyphosate, no detectable levels were found neither in GBH LD nor in GBH HD treated dams. Finally, no glyphosate or AMPA levels were detected in control animals. Similar results were obtained on GD22 (data not shown). All these data are shown in Table 3.

Effect of perinatal GBH treatment on body weight and vaginal opening in F1 females

In both treatment groups, the body weight and the onset of vaginal canal opening were similar to control at each of the studied points (Fig. 2A and B).

Fig. 2*: Effect of perinatal GBH treatment on weight and onset of vaginal canal opening in F1 female rats. (A) Body weight curves of control and perinatally exposed F1 female rats to GBH LD or GBH HD through diet until adulthood. (B) Onset of vaginal canal opening expressed as post-natal day (PND) for each experimental group. All data are presented as mean ± SEM (Control, n = 25, GBH LD, n = 20, GBH HD, n = 20).



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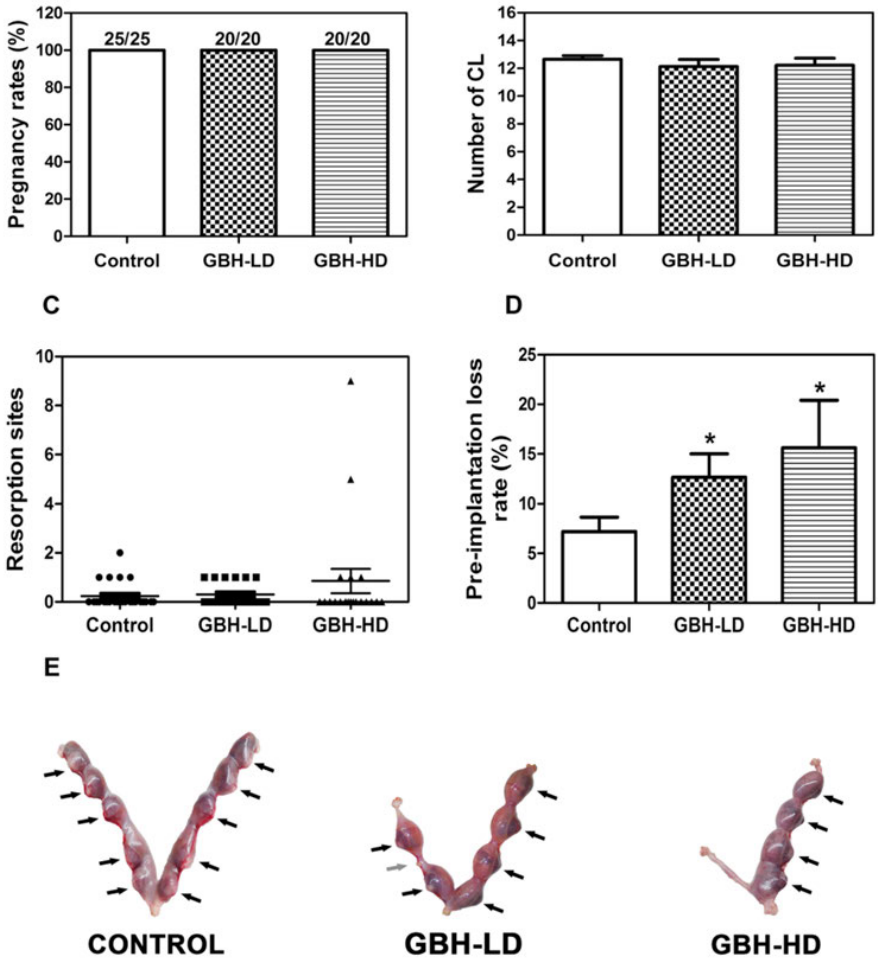
Reproductive performance of perinatally GBH exposed F1 females on GD19

Pregnancy rates, CLs or the number of resorption sites were not affected in F1 females by the treatment. However, both treatment groups showed a lower number of implantation sites with a significant increase of pre implantation loss. A peculiar effect was found in some of the F1 females from GBH LD and GBH HD groups, which presented unilateral pregnancies. In fact, in 2 out of 20 rats from GBH LD group and 3 out of 20 rats from GBH HD group, embryo implantation occurred only in one uterine horn, even though the number of CLs in the ovary adjacent to the no pregnant uterine horn was normal. However, these findings did not show to be statistically significant. All results are shown in Fig. 3.

Fig. 3*: Evaluation of reproductive performance in control and GBH perinatal exposed F1 rats recorded on gestational day 19 (GD19). (A) The pregnancy rates were calculated by the average of females that

were pregnant with a fertile male. (B) The number of CLs and (D) preimplantation loss rate is expressed as the mean ± SEM for each experimental group (Control, n = 25; GBH LD, n = 20; GBHHD, n = 20). (C) The numbers of resorption sites in each individual pregnant rat were plotted, and the horizontal lines are the mean for each group (n ≥20 per group) with its corresponding SEM. Asterisks indicate statistical significance compared to the control (*, p <0.05). (E) Photographs of representative uteri collected on GD19 from a control and a GBH LD or GBH HD perinatally exposed F1 female rat. Note the lower number of implantation sites in the uterus of a GBH LD and GBH HD F1 rat compared to a control rat. Each black arrow indicates an implantation site, and the grey arrow indicates a resorption site.



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Feto placental parameters of F2 offspring1

F2 fetuses (fetuses/litters: Control, n = 213/20; GBH2, n = 152/15; GBH200, n = 117/13) from F1 females exposed to both doses of GBH showed a decrease in length and body weight (Table and Fig. 4).

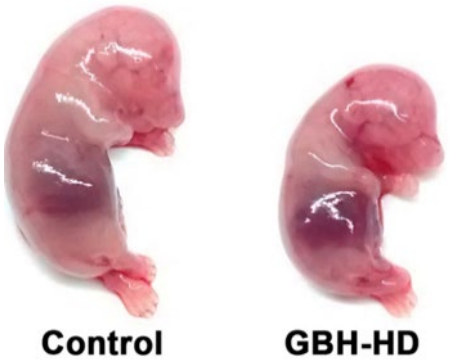
Table 4*: Feto placental parameters of F2 offspring from control and GBH exposed F1 female rats

	Control (*n*: 213 fetuses/20 litters)	GBH-LD (*n*: 152 fetuses/15 litters)	GBH-HD (*n*: 117 fetuses/13 litters)
Fetal length (cm)	2.44 ± 0.01	2.39 ± 0.01*	2.29 ± 0.02***
Fetal weight (mg)	1307.97 ± 12.84	1227.43 ± 15.78***	1212.05 ± 20.01***
Placental weight (mg)	359.76 ± 3.90	349.17 ± 6.56	390.23 ± 11.22**

Data are shown as mean ± SEM. All differences statistically significant are indicated as *($0.05 > p > 0.01$); **($0.01 > p > 0.001$); ***($p < 0.001$) from control group [based on one-way analysis of variance (ANOVA) with Dunnett's post hoc test]

*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

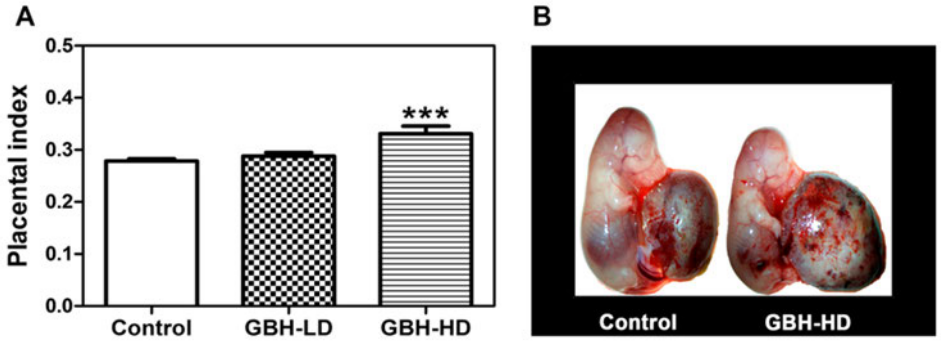
Fig. 4*: Fetal parameters of F2 offspring from control and GBH perinatally exposed F1 female rats. Photograph of representative F2 offspring showing lower fetal length and weight in fetuses from GBH HD F1 females in comparison to control group.



*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

From the weighted frequency distribution curve, 57.5% and 48.2% of the F2 fetuses from GBH LD and GBH HD groups, respectively, were SGA fetuses, i.e., with a weight < 10th percentile. In the control group, only 23.7% of the fetuses were SGA. In addition to low weight and length, F2 fetuses from F1 females perinatally exposed to GBH HD exhibited higher placental weight (Table 4) and placental index (Fig. 5A). Differences in size of F2 fetuses and their corresponding placentas from control and GBH HD group can be appreciated in Fig. 5B.

Fig. 5*: Placental parameters of F2 offspring from control and GBH perinatally exposed F1 female rats. (A) Placental index is expressed as mean ± SEM. (B) Photographs of representative F2 offspring showing lower fetal weight and higher placental weight in fetuses from GBH HD F1 females than control animals.



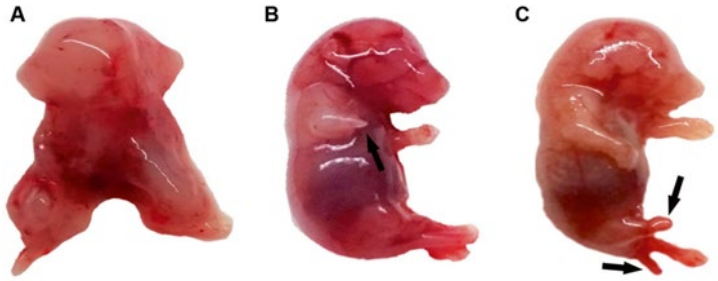
*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

Fetal morphology of F2 offspring

Fetal malformations were detected in some of the F2 fetuses from mothers perinatally exposed to the high dose of GBH (200 mg/kg bw/day). Fetal abnormalities were observed in 3/117 fetuses, each one from different F1 mothers (i.e., 3/13 litters affected). A statistically significant correlation was found between perinatal GBH HD exposure and fetal anomalies ($p < 0.05$). These anomalies included conjoined fetuses and abnormally developed limbs (Fig. 6). The last case included fetuses, which lacked one of their extremities or exhibited a longitudinal reduction of the tail (Fig. 6B, C). No structural anomalies were observed in F2 fetuses from GBH LD or control mothers.

Fig. 6*: Foetal morphology of F2 offspring from GBH HD exposed F1 female rats. In the photographs are shown structural congenital anomalies, such as (A) conjoined foetuses and abnormally developed limbs, in (B) a foetus lacking one of its anterior extremities (indicated by the black arrow) and in (C) a foetus

lacking one of its posterior extremities and displaying a longitudinal reduction of the tail (indicated by black arrows). Three structural anomalies were detected in 117 GBH HD F2 foetuses analysed (3/117) in (3/13) litters (*, p <0.05)



*Retrived from Milesi, M.M., *et al*., Archives of Toxicology (2018) 92: 2629-2643

Conclusion
The present study investigates the effects of in utero and lactational exposure of 2 and 200 mg/kg bw/day of a GBH, administered orally to F0 mothers, on the development and reproductive outcome of F1 female rats. Further, fetoplacental parameters of F2 offspring were evaluated to assess possible second generation effects. The results demonstrated that perinatal exposure to low doses of a GBH alters the female reproductive outcome and induces second generation adverse effects in rats. The most salient results include: (1) impaired reproductive capability characterized by an increase in the rate of preimplantation embryo loss in F1 females, and (2) fetal growth retardation and structural congenital anomalies in their progeny (F2 generation). The present and previous findings suggest that GBHs might act as endocrine disrupters, and highlight the importance of assessing different administration routes, doses and window/lengths of exposure to GBHs since the effects on fertility can be different.

Assessment and conclusion

Assessment and conclusion by applicant:
Relevant but supplementary information: Glyphosate based herbicide (54% w/v glyphosate acid equivalent as potassium salt) dosed to pregnant rats. Only 2 doses of the test substance used.

The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains potassium salt, thus the composition differs to the EU representative formulation MON 52276. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Further points for clarification:
This study investigates the effects of a glyphosate based herbicide on the reproductive performance in rats and the consequence of exposure during the in utero and lactation phases on the subsequent generation. However, an insufficient number dose groups and an insufficient number of F0 treatment animals (n = 7) resulted in less than half the number of required litters for an adequate data set as described in OECD Developmental and Reproductive Toxicology Test Guidelines.

Pregnant rats were administered a glyphosate based herbicide via the diet at 2 or 200 mg/kg bw/day from gestational day (GD) 9 until weaning of their offspring (F1).

The following parameters were analysed and none of them was affected by the treatment: length of gestation, birth weight of male and female pups, litter size and maternal care.

Further, sexually mature F1 females were mated and allowed to litter. These animals were not directly dosed with glyphosate formulation. The reproductive performance was unaffected regarding pregnancy rates, CLs or the number of resorption sites. However, F1 from F0 treated with 2 and 200 mg/kg bw/day showed a lower number of implantation sites with a significant increase of pre implantation loss and the respective F2 offspring showed reduced growth/weight and structural congenital anomalies.

It is difficult to interpret and conclude on the relevance of these data. F0 were clearly exposed to systemic glyphosate, and there were no effects on the reproductive parameters measured and on the F1 growth and development. However, F1 were not given glyphosate formulation and no blood analysis was carried out to check for eventual systemic concentration of glyphosate or AMPA. So it is difficult to correlate any potential observed effect to glyphosate.

The rats in the study were derived from Wistar rats and are a university-owned inbred colony. There is no historical data, provided or publicly available information for these rats. Such information is critical to enable a comparison of measure outcomes, particularly with the insufficient number of litters available for evaluation. For example, in the absence of historical data, there is no information about the background incidence of abnormalities for the colony used in this paper. Based on available information provided in the literature for Wistar rats, it appears that foetal lengths, weights, and placental weights are all with normal ranges (see references no. 1 and 2, below). The differences noted in this study between the control and treatment groups are small and within normal ranges.

Moreover, no historical control data were presented to support the interpretation/relevance of the observed structural malformation in F2 data.

Overall, the relevance of this information is doubtful.

References:
1. Norman, N.A. and Bruce, N.W. Fetal and placental weight relationships in the rat at days 13 and 17 of gestation. J Reprod Fertil, 1979. 57(2): p. 345 8.
2. Rahima, A. and Bruce, N.W. Spacing of conceptuses in the uterine horn and local effects on fetal and placental weights throughout gestation in the rat. J Reprod Fertil, 1986. 78(2): p. 741 7.

Assessment and conclusion by RMS:
In this study seven pregnant females (F0) per group were administered a glyphosate-based herbicide (GBH) mixed with laboratory pellet chow based paste in a dose of 2 and 200 mg of glyphosate/kg bw/day from gestational day 9 until weaning. Additionally, a control group consisting of seven pregnant females treated with the same diet but without glyphosate was included. The serum concentrations of glyphosate and AMPA were determined at the end of the lactation period. Body weight gain and vaginal canal opening of F1 females were recorded. Sexually mature F1 females were mated and their reproductive performance assessed by determination of pregnancy rate and on gestational day 19, the number of corpora lutea, the implantation sites and resorption sites. Second generation effects on the F2 offspring, foetal morphology on gestational day19, foetal length and weight, and the placental weight were analysed.

GBH exposure was not found to alter the body weight gain, vaginal opening onset, pregnancy rates, number of corpora lutea or resorption sites of F1 females. However, the number of implantation sites were lower in F1 females of both exposed groups with an increased pre-implantation loss.

F2 offspring from both GBH exposed groups showed lower foetal weight and length. Also a higher incidence of small for gestational age foetuses, higher placental weight and structural congenital anomalies (conjoined foetuses and abnormally developed limbs) were found in F2 offspring from 200 mg/kg bw/day treated dams.

Comments on the paper to the editor of the journal
It should be noted that Plewis (2019) in a letter to the editor claims that the article by Milesi et.al. (2018) suffers from serious methodological defect in that the authors do not appear to take into account “litter” effects in their analysis. The offspring of a particular female rat are likely to be more alike than the offspring of different rats. Hence, the assumption in the statistical analysis that both first and second generation rats are independent samples in each of the three experimental groups is incorrect. In fact, the first generation sample is clustered by their parents and the second generation is clustered by both their parents and grandparents. Ignoring this clustering can lead to incorrect statistical interferences. In particular it is likely that the p values quoted in the paper are far too low, especially for the F2 rats. In other words, the differences observed at second generation could be just chance differences.

Following the comment by Plewis (2019), Milesi et.al. reanalysed the data and took account of dependence of

offspring from the same dam. However, no statistically significant difference between the 2 mg/kg bw/day group and the control group was found which is out of line with Fig. 3d. In table 2, there is no longer any treatment effect for placental weight or for foetal length for the 2 mg/kg bw/day group.

Plewis (2020) concluded that the reanalyses do not go far enough in terms of allowing for the hierarchical structure of the data and assertions that their original findings hold up after the reanalyses are not borne out. It is plausible to suppose that allowing for the full hierarchical structure would lead to a further reduction in the precision of the estimated treatment effects.

References:
- Milesi, M.M, Lorenz, V., Beldomenico, P.M., Vaira, S., Varayoud, J. and Luque, E.H. (2019). Comment on response from Milesi *et al*. to 'Perinatal exposure to a glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats'. 93, 3635–3638
- Plewis, I. (2019). Comment on: Perinatal exposure to a glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats. Arch. Toxicol. 93, 207
- Plewis, I. (2020). Comment on response from Milesi *et al*. to 'Perinatal exposure to a glyphosate-based herbicide impairs female reproductive outcomes and induces second-generation adverse effects in Wistar rats'. Arch. Toxicol. 94, 351-352

RMS' conclusion
In conclusion, perinatal exposure to 2 and 200 mg of glyphosate/kg bw/day from gestational day 9 until weaning lowered the number of implantation sites in F1 females with an increased pre-implantation loss and may induce foetal growth retardation and structural congenital anomalies in F2 offspring.

The study is considered as supplementary data only. The study is carried out with a formulation of glyphosate, thus effects caused by co-formulants cannot be excluded. The study is not reliable due to uncertainties with regard to statistical analyses. Furthermore, the test substance is not sufficiently characterised (particularly, purity and batch not specified), small group sizes of animals used, inbred strain of animals used, no details on food, clinical observations not presented, no historical control data and positive control missing. It could also be noted that the rats were not exposed during the whole period of organogenesis since exposure started at GD 9 instead of GD 5 as recommended in the OECD TG 414.

B.6.6.3.2. Public literature relevant for section B.6.6.2

Summary of published literature studies identified by the applicant as relevant and reliable or reliable with restrictions (Category A):

Category A – Yahfoufi, Z.A. *et al.*

Data point	CA 5.8.2
Report author	Yahfoufi Z. A. *et al*.
Report year	2020
Report title	Glyphosate Induces Metaphase II Oocyte Deterioration and Embryo Damage by Zinc Depletion and Overproduction of Reactive Oxygen Species
Document source	Toxicology, (2020) Vol. 439, Art. No. 152466
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary/reliable with restrictions (see assessment and conclusion by RMS)

Aim of the study

The aim of the study was to investigate the effects of glyphosate (0 – 300 µM) on metaphase II mouse oocyte quality and embryo damage to obtain insight on its mechanisms of cellular action and the tolerance of oocytes and embryos towards glyphosate.

Materials and methods

Test material:	Glyphosate as powder from Research Products International (Mt. Prospect, IL, USA; catalog G36060, CAS 1071-83-6)
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Not applicable
Mouse *in vitro*	Cryopreserved metaphase II noncumulus oocytes and embryos from a B6C3F1 mouse crossed with a B6D2F1 mouse from Embryotech Inc. MA, USA
Materials	Metaphase II noncumulus oocytes and embryos from a B6C3F1 mouse crossed with a B6D2F1 mouse were obtained commercially from Embryotech Inc. in cryopreserved straws using the ethylene glycol-based slow freeze cryopreservation protocol. Human tubular fluid (HTF) medium with gentamicin was obtained from Irvine Scientific Inc. Anti-α tubulin antibody, fluorescein isothiocyanate (FITC) conjugate anti-goat antibody, 4′,6′-diamino-2-phenylindole (DAPI), and 0.1% Triton X-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). The anti-fade agent, formaldehyde, SYTO 64 Red Fluorescent Nucleic Acid Stain, Alexa Fluor® 633 goat anti-mouse IgG, and dimethylsulfoxide (DMSO) were obtained from Thermo Fisher Scientific Inc. The Purified Mouse Anti-Mouse Pericentrin was purchased from BD Biosciences and Fab Fragment Affinity Purified Antibody was purchased from Jackson ImmunoResearch Inc (West Grove, PA, USA). The cellular reactive oxygen species (ROS) detection assay kit and zinc detection kit (Zinquin ethyl ester) were obtained from Abcam (Cambridge, UK). Glyphosate (C3H8NO5P, powder form) was purchased from Research Products International (Mt. Prospect, IL, USA; catalog G36060, CAS 1071-83-6).

Methods

Frozen mouse metaphase II oocytes and embryos were obtained from Embryotech (MA, USA). The concentrations of glyphosate treatment used here ranged from 0 to 300 µM.

Study the effect of glyphosate on MT and CH alignment of metaphase II noncumulus oocytes (total number of oocytes ≈ 180)

Oocytes were thawed and transferred from straws to phosphate buffer saline (Dulbecco PBS) and washed for 3 min to remove cryo-protectant. Oocytes were then transferred to HTF media and incubated at 37°C and 5% carbon dioxide (CO_2) for 60 min to allow spindle re-polymerization and attainment of normal oocyte architecture. The oocytes were then screened for the presence of the polar body confirming their Metaphase II stage. Immature oocytes or those that displayed disrupted zona pellucida were discarded.

In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average; total ~180 oocytes used across replicates) were exposed to 0, 25, 50, 75, 100, 200, and 300 µM glyphosate for a 2 h incubation period (37°C, 5% CO_2) to observe the effects on oocyte microtubule (MT) and chromosomal (CH) alignment. Only some concentrations were selected for presentation to improve focus. Untreated oocytes served as controls.

Immunostaining of the oocytes and confocal microscopy examination

Oocytes were subsequently fixed in a solution prepared from 2% formaldehyde and 0.2% Triton X-100 for 30 min at 25°C, then washed with PBS for 3 min. Subsequently, the oocytes were subjected to indirect immunostaining in mouse primary anti-α-tubulin antibody (1:300) against the MT overnight at 25°C, then washed and placed in secondary FITC conjugated anti-goat antibody (1:50) for 45 min at 25°C. After washing in PBS, the chromosomes were stained using DAPI (1:100) for 15 min at 25°C. For immunostaining of pericentrin, oocytes were treated with primary antibody, Purified Mouse Anti-Mouse Pericentrin (1:100), overnight at 4°C prior to staining with secondary antibody, Alexa Fluor® 633 goat anti-mouse IgG (1:200) for 2 h at 25°C. Oocytes were then washed 3 times before immunoglobulin blocking with Fab Fragment Affinity Purified Antibody (20 µg/ml) for 30 min at 25°C, prior to washing and staining for MT and chromosomes.

Stained oocytes were loaded into anti-fade agent on slides with two etched rings and cover slips placed using transparent nail varnish. Slides were stored at −20°C and protected from light until they were evaluated for more details by confocal microscopy. To obtain confocal images, slides were examined using a Zeiss LSM 510 META NLO microscope using FITC (green), DAPI (blue), and Alexa Fluor® 633 (bright red) fluorescent filters with excitation and emission wavelengths of 495 and 519 nm, 358 and 461 nm, and 632 and 647 nm, respectively. Oocytes were localized using a 10x magnification lens and spindle alterations were assessed using 40x oil immersion lens. The MT was stained fluorescent green, which was distinct from the fluorescent blue staining of chromosomes. Fluorescence images were saved as graphic files in TIFF format. The fluorescence intensity of each oocyte was quantified and analysed with Image J software, version 1.41o (National Institutes of Health, Bethesda, MD, USA). We used the following formula to calculate the corrected total cell fluorescence (CTCF): (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings). The calculated CTCF was normalized by cell area of the respective sample and presented as normalized CTCF. Student's t-test was performed on the CTCF to test if the difference between control and each glyphosate treatment group was statistically significant ($p<0.05$).

Assessment of MT and CH Alignment (Scoring System)
Each treated and control oocyte from every experiment set was closely examined for spindle and chromosome configurations. Three independent observers who were blinded to treatment group assignment and used comprehensive evaluation of the individual optical sections performed the categorization of oocytes based on MT and CH configurations.

Three observers scored the oocytes and compared the alterations in the MT and CH alignment to controls. Scores of 1-4 were assigned for both MT and CH alterations, with good outcomes associated with scores 1 or 2 and poor outcomes associated with scores 3 or 4. Good spindle configurations (score 1 or 2) generally exhibit barrel-shaped microtubules, whereas poor or abnormal configurations (score 3 or 4) display shortened spindle length, disorganization, or completely missing spindles. Good chromosomal configurations (score 1 or 2) generally exhibit chromosomes arranged at the equator of the spindle, while poor chromosomes (score 3 or 4) appear dispersed, irregular, or less condensed.

Detection of intracellular zinc in oocytes and embryos after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)
In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared, and pretreated with increasing concentration of glyphosate for 2 h (0, 25, 50, 75, 100, 200, and 300 μM). Subsequently, oocytes were stained with 25 μM Zinquin ethyl ester in combination with 1 μM SYTO64 and incubated for 1 h at 37°C, 5% CO_2. A single dilution of Zinquin ethyl ester was used in each dish to ensure that all dishes received the same concentration of dye. This zinc-selective fluorescent probe permeates the cell and labels the intracellular zinc ions to emit a quantifiable blue fluorescent signal. Cells were then washed in PBS for 3 min to remove any excess dye before fixation with 2% formaldehyde and 0.2 % Triton X-100 for 15 min at 25°C. After washing, cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Confocal microscopy examination and assessment of intracellular zinc
Oocytes and embryos were examined to obtain confocal images using a Zeiss LSM 510 META NLO microscope equipped with a stage top incubator, using Texas Red (red) and DAPI (blue) fluorescent filters with excitation and emission wavelengths of 480 and 535 nm, and 358 and 461 nm, respectively. Fluorescence microscopy images were taken using a Nikon Eclipse 90i epifluorescence microscope and were saved as graphic files in TIFF format. The fluorescence intensity of each oocyte was quantified and analysed with Image J software, version 1.41o (National Institutes of Health, Bethesda, MD, USA). We used the following formula to calculate the corrected total cell fluorescence (CTCF): (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings). The calculated CTCF was normalized by cell area of the respective sample and presented as normalized CTCF. Student's t-test was performed on the CTCF to test if the difference between control and each glyphosate treatment group was statistically significant ($p<0.05$).

Detection of ROS generation in oocytes after glyphosate exposure (total number of oocytes ≈ 180; total number of embryos ≈ 180)
In triplicate experiments, noncumulus oocytes (n = 7 oocytes per group on average) were thawed, prepared, and exposed to 0, 25, 50, 75, 100, 200, and 300 μM glyphosate as outlined above in detail (under "oocyte preparation"). Oocytes were incubated at 37°C, 5% CO_2 in glyphosate treatment for 2 h and in 100 μM

pyocyanin for 45 min. Oocytes treated with pyocyanin served as positive control, while untreated oocytes served as negative control. The generation of ROS was evaluated using the Cellular ROS Detection Assay kit obtained from Abcam (Cambridge, UK). The kit selectively tests for the generation of multiple ROS products such as hydroxyl radical and superoxide and the resulting changes in fluorescent intensity of the probe in the presence of those products. The cell-permeant reagent, ROS Deep Red dye reacts with ROS to produce the bright fluorescent signal that is quantified and compared to control.

After incubating with the treatment, oocytes were stained with 500 µL volume of ROS deep red working solution (a mixture of 250 µL HTF media, 249 µL ROS buffer, and 1 µL ROS Deep Red dye) and incubated for 1 h at 37°C, 5% CO_2 before subsequent washing in PBS. After fixation by 2% formaldehyde and permeabilization with 0.2% Triton X- 100 for 30 min, cells were washed and nuclei were lastly stained with DAPI (1:100) for 15 min at 25°C. Cells were loaded into anti-fade agent on slides for subsequent evaluation by confocal microscopy. A similar procedure was used for embryos (n = 7 embryos per group on average).

Confocal microscopy examination and assessment of ROS generation
Oocytes and embryos were examined to obtain confocal images using a Zeiss LSM 510 META NLO microscope equipped with a stage top incubator, using Texas Red (red) and DAPI (blue) fluorescent filters with excitation and emission wavelengths of 480 and 535 nm, and 358 and 461 nm, respectively. Images of ROS-mediated deep red fluorescence were taken using a Nikon Eclipse 90i epifluorescence microscope. Fluorescence images were saved as graphic files in TIFF format. The fluorescence intensity of each oocyte was quantified and analysed with Image J. We used the previously described formula to calculate the CTCF, (CTCF = Integrated Density – (Area of selected cell X Mean fluorescence of background readings) (Sun *et al*. 2016). The calculated CTCF was normalized by cell area of the respective sample and presented as normalized CTCF. Student's t-test was performed on the CTCF to test if the difference between control and each glyphosate treatment group was statistically significant ($p<0.05$).

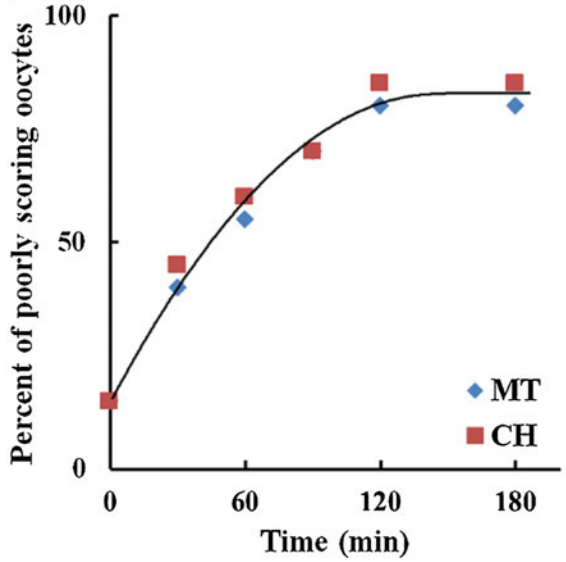
Statistical analysis
The MedCalc statistics program (Version 7.2.1.0, 2003; MedCalc Software, Mariakerke, Belgium) was used to perform all statistical analyses. Comparisons of the percentage of oocytes with poor scores (scores 3 and 4) for MT and CH were made using one-tailed z-tests, with continuity correction, to evaluate if each treatment group had a greater proportion of poor scores compared to the control. Comparisons of normalized CTCF between each treatment group and control were performed by Student's t-test. $p<0.05$ was considered significant for all statistical tests.

Results

Glyphosate altered MT (oocyte microtubule) and CH (chromosomal) alignment of metaphase II mouse oocytes
To justify the 2 h incubation time period, glyphosatetreated (100 µM) oocytes were incubated at 37°C, 5% CO_2 for 30, 60, 90, 120, and 180 min and untreated control oocytes for 180 min (n = 20 for each). The time-dependent effect was examined and plotted the percentage of poorly scored oocytes as a function of time and compared to control. The untreated oocytes displayed the same normal spindle morphology throughout the 180 min incubation time, however, the treated oocytes displayed maximum MT and CH alterations (80-85%) at 120 min and remained unchanged beyond this point. Fig. 1 shows a representative sample of poorly scored glyphosate-treated oocytes as a function of time. The percentage of poor scores for MT and CH alignment increases over time and reaches a maximum effect at 120 min, justifying the 2 h incubation period we chose to use for all subsequent analyses.

Fig. 1*: The time-dependent effect of glyphosate on oocyte quality
A) The percent of oocytes with poor scores in MT structure and CH alignment at different exposure times (0 to 180 min) when oocytes (n = 20 per group) were incubated with a fixed concentration of glyphosate (100 µM) and compared to untreated control that were incubated for 180 min (represented at the point on the Y axis). Oocytes were then visualized via immunofluorescence staining. B) The mean of percentage of poorly scoring oocytes for each exposure time were represented with the respective standard error of the mean.

A)



B)

Time of exposure (min)	Percent of poorly scoring oocytes (Mean ± SE) MT	CH
0*	15 ± 2	15 ± 3
30	40 ± 5	45 ± 6
60	55 ± 7	60 ± 3
90	70 ± 5	70 ± 8
120	80 ± 7	85 ± 5
180	80 ± 7	85 ± 7

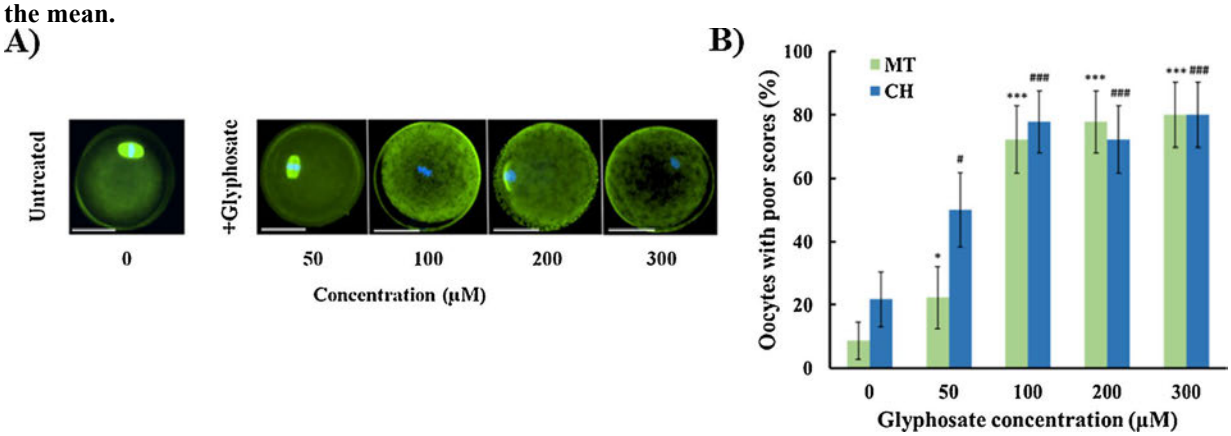
*Control oocytes received no treatment and were incubated for 180 min

*Retrived from Yahfoufi, Z. A., *et al*., Toxicology 439 (2020) 152466

The effect of increasing concentrations of glyphosate was investigated on oocyte quality as demonstrated by alteration in MT and CH alignment. Oocytes were incubated with increasing concentrations (50, 100, 200, and 300 µM) of glyphosate for 2 h. Fig. 2A shows representative images of untreated and treated oocytes, while Fig. 2B shows the quantification of percent of poor scoring oocytes as a function of glyphosate concentration based on a previously published 1–4 scoring system. Untreated oocytes had a well-organized barrel shaped spindle structure (green) with chromosomes tightly aligned at the equatorial plate of the spindles (blue) (Fig. 2A). Oocytes exposed to low glyphosate concentration had altered spindle configuration indicated by an enlarged "balloon" shape and some disturbance in the chromosome configuration, while oocytes treated with high glyphosate concentration (> 100 µM) exhibited a scattered pattern of chromosomes and missing microtubules. As shown in Fig. 2B, the damaging effects of glyphosate on both MT and CH quality were exhibited by the significant increase in the quantity of oocytes with poor scores when exposed to higher concentrations of glyphosate compared to control ($p<0.001$). The control group had approximately 8.69% and 21.7% poor scores for both MT and CH scoring, respectively. The remaining percentage of oocytes with poor MT scores were 22.2% ($p<0.05$), 72.2%, 77.7%, and 80% ($p<0.001$) and poor CH scores were 50% ($p<0.05$), 77.7%, 72.2%, and 80% ($p<0.001$) for 50, 100, 200, and 300 µM glyphosate, respectively. Overall, the results demonstrate that increasing glyphosate concentration induces damage to MT and CH alignment as well as substantial oocyte deterioration exhibited beyond 100 µM.

Fig. 2*: The effect of glyphosate on metaphase II mouse oocyte spindles and chromosomes
A) Representative fluorescent confocal images of metaphase II mouse oocytes stained with α-tubulin antibody to visualize microtubules (MT) (green) and counterstained with DAPI to visualize chromosomes (CH) (blue). After incubating for 2 h, exposing oocytes to increasing concentrations of glyphosate (25, 50, 75, 100, 200 and 300 µM; though 25 and 75 µM were omitted here to improve focus) corresponded with appearance of various abnormal spindle configurations, compared to the untreated control (n = 7/group). Scale bars: 35 µm. Images shown are from a typical triplicated experiment. B) The percentages of oocytes with poor scores (3 and 4) in MT structure and CH alignment increase with increasing concentration of glyphosate; treatment groups with percent of poorly scored oocytes that were statistically significantly greater than the control are indicated by * for MT and # for CH (* indicates $p<0.05$, * indicates $p<0.001$; # is used similarly; z-test with continuity correction). The experiment was conducted in**

triplicate, and scores were assigned by three independent observers. Error bars indicate standard error of the mean.

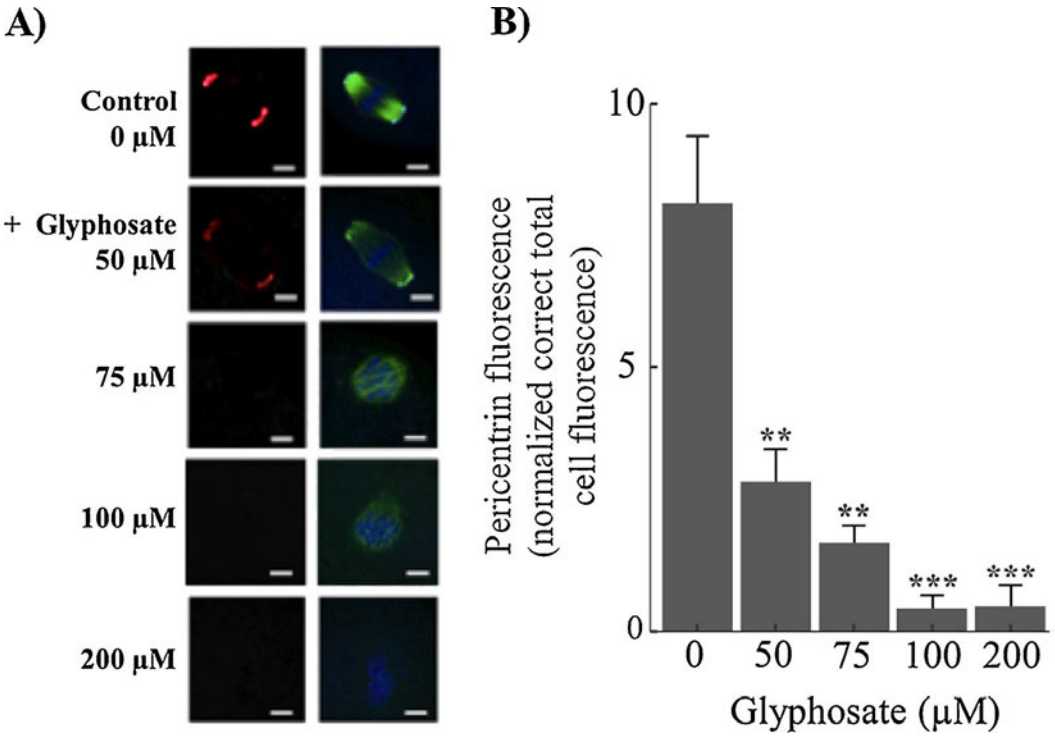


*Retrived from Yahfoufi, Z. A., *et al*., Toxicology 439 (2020) 152466

Glyphosate mediates pericentrin disappearance

To investigate the effect on MT and CH alignment, specifically the mechanisms causing meiotic spindle disappearance, the impact of increasing glyphosate concentration on MTOC (microtubule organizing center) components were evaluated. Fig. 3 shows representative images of untreated and glyphosate-treated metaphase II mouse oocytes (50, 75, 100, and 200 µM). Control oocytes had normal pericentrin (bright red) localized at each spindle pole, maintaining the spindle force balance with a well-organized MT structure (green) and chromosomes (blue) tightly aligned at the equatorial plate. Low treatment groups (50 µM) displayed pericentrin proteins moving closer together from each spindle pole, while remaining within the polar axis. However, at higher glyphosate concentrations (> 75 µM), pericentrin disappears disrupting the spindle force balance and causing chromosomal dispersion and spindle loss. As seen in Fig. 3B, pericentrin is diminished significantly at 50 µM ($p<0.01$), and the effect increases dramatically at 100 µM and higher ($p<0.001$). Taken together, these findings demonstrate that increasing glyphosate concentration can cause substantial pericentrin damage, thus influencing MT and CH alignment and morphology.

Fig. 3*: The effect of glyphosate on the structure of pericentrin in metaphase II mouse oocytes
A) Representative fluorescent confocal images of metaphase II mouse oocytes stained for spindles (green, anti-α-tubulin antibody), chromosomes (blue, DAPI) and pericentrin (bright red, Alexa Fluor® 633) when treated with increasing concentrations of glyphosate (0, 25, 50, 75, 100, 200, and 300 µM; some treatments omitted from figure for greater focus). After 2 h incubation, oocytes were fixed and evaluated for pericentrin localization and microtubule organizing center (MTOC) status. As glyphosate concentration increases, pericentrin presence is increasingly diminished, until it vanishes at 75 µM and greater concentrations. Scale bar: 5 µm. Images shown are from a typical experiment performed in triplicate. B) Quantification of pericentrin fluorescence in metaphase II mouse oocytes treated with increasing concentrations of glyphosate (0-300 µM). Fluorescence was quantitated in terms of corrected total cell fluorescence (CTCF), normalized for spindle area, and presented as mean±SEM (Standard error of the mean). Statistical significance was indicated by **, * ($p<0.01$, 0.001; Student's t-test). Error bars represent standard error of the mean.**

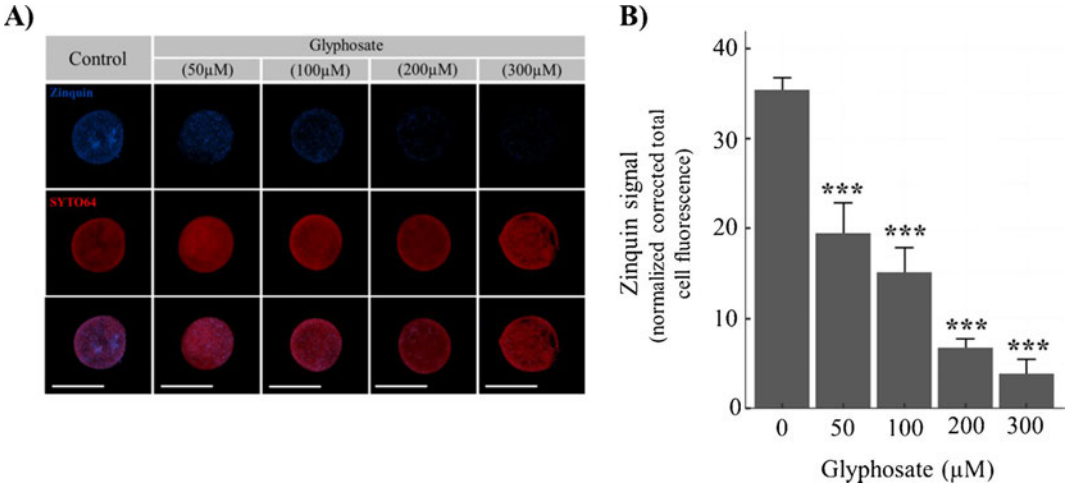


*Retrived from Yahfoufi, Z. A., *et al*., Toxicology 439 (2020) 152466

Glyphosate reduced intracellular zinc content

Oocytes were exposed to increasing glyphosate concentrations of 50, 100, 200 and 300 µM for 2 h and the intracellular zinc content was assessed by using Zinquin ethyl ester, a zinc sensitive and cell–permeable fluorescent probe. Increasing glyphosate concentration correlated with zinc depletion as indicated by a decrease in blue fluorescent intensity compared to untreated control, which is likely attributed to glyphosate's chelating ability (Fig. 4). Fig. 4A shows representative images obtained by confocal microscopy of the different glyphosate concentrations tested (50-300 µM). Normalized CTCF was calculated for the groups tested (Fig. 4B). The difference between the CTCF of each treatment group compared to the untreated control was found to be statistically significant ($p<0.001$). Oocytes treated with 300 µM had the lowest zinc-mediated fluorescence, while oocytes treated with 50 µM had the highest fluorescence with an average normalized CTCF of 3.8±1.6 and 19.4±3.4, respectively (mean± SEM) (Fig. 4B).

Fig. 4*: Evaluation of zinc reduction in oocytes exposed to increasing glyphosate concentration
A) Representative fluorescent confocal images of metaphase II mouse oocytes stained for zinc (blue, Zinquin ethyl ester) and nucleic acid (red, SYTO64) when treated with increasing concentration of glyphosate (0, 25, 50, 75, 100, 200 and 300 µM; some treatments omitted from figure for greater focus). Nucleic acid staining was used to indicate the presence of the cells. Note the gradual decrease in zinc content as glyphosate concentration increases. Scale bars: 75 µm. Images shown are from a typical experiment performed in triplicate. B) Quantification of zinc fluorescence in metaphase II mouse oocytes treated with increasing glyphosate concentration (0-300 µM). Fluorescence was quantitated in terms of corrected total cell fluorescence (CTCF), normalized for cell area, and presented as mean±SEM (Standard error of the mean). Statistical significance was indicated by * ($p<0.001$; Student's t-test). Collectively, these results display that glyphosate deteriorates oocyte quality by chelating intracellular zinc content.**



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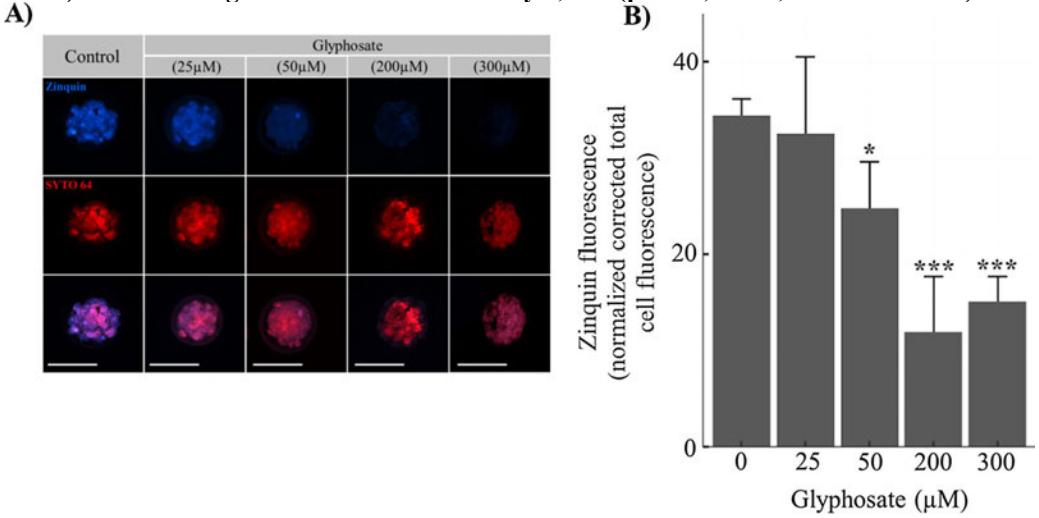
The effect of glyphosate exposure on intracellular zinc content in embryos exposed to increasing glyphosate concentrations of 50, 100, 200, and 300 µM for 2 h was assessed to examine if glyphosate's ability to remove zinc was also present in embryos. Similar to oocytes, intracellular zinc declined with glyphosate treatment (Fig. 5). Fig. 5B shows that 50 µM glyphosate treatments reduced intracellular zinc by 37%, and increasing the concentration of glyphosate maintained this effect ($p<0.01$ for comparisons of treatment groups with the control).

Fig. 5*: Evaluation of zinc reduction in embryos exposed to increasing glyphosate concentration
A) Representative fluorescent confocal images of mouse embryos stained for zinc (blue, Zinquin ethyl ester) and nucleic acid (red, SYTO64) when treated with increasing concentration of glyphosate (0, 25, 50, 75, 100, 200, 300 µM; some treatments omitted from figure for greater focus). Nucleic acid staining was used to indicate the presence of the cells. Scale bars: 100 µm. Images shown are from a typical experiment performed in triplicate. B) Quantification of zinc fluorescence in mouse embryos treated with increasing glyphosate concentration (0-100 µM). Fluorescence was quantitated in terms of corrected total cell fluorescence (CTCF), normalized for cell area, and presented as mean±SEM (Standard error of the mean). Statistical significance was indicated by *, * ($p<0.05$, 0.001; Student's t-test).**



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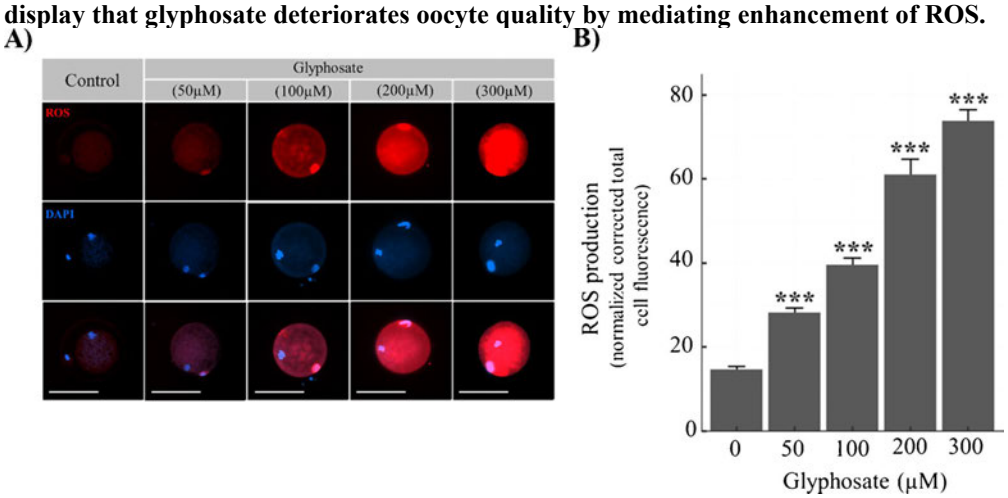
Glyphosate increased ROS generation
To test whether glyphosate exposure increases ROS generation in metaphase II oocytes, oocytes were exposed to increasing glyphosate concentrations of 50, 100, 200 and 300 µM for 2 h. Intracellular ROS generation (superoxide and hydroxyl radical) was assessed by using the cellular ROS detection assay kit from Abcam. Increasing glyphosate concentration correlated with an increase in ROS formation as signified by an increase in deep red fluorescence compared to negative control (Fig. 6). Fig. 6A shows representative images obtained by confocal microscopy for 50 and 100 µM glyphosate concentrations. Normalized CTCF was calculated for the

groups tested (Fig. 6B). The difference between the CTCF of each treatment group compared to the negative control was found to be statistically significant ($p<0.001$). Oocytes treated with 50 µM had the lowest ROS fluorescence, while oocytes treated with 300 µM had the highest ROS fluorescence with an average normalized CTCF of 28.3±1.1 and 73.8±2.6, respectively (mean±SEM) (Fig. 6B).

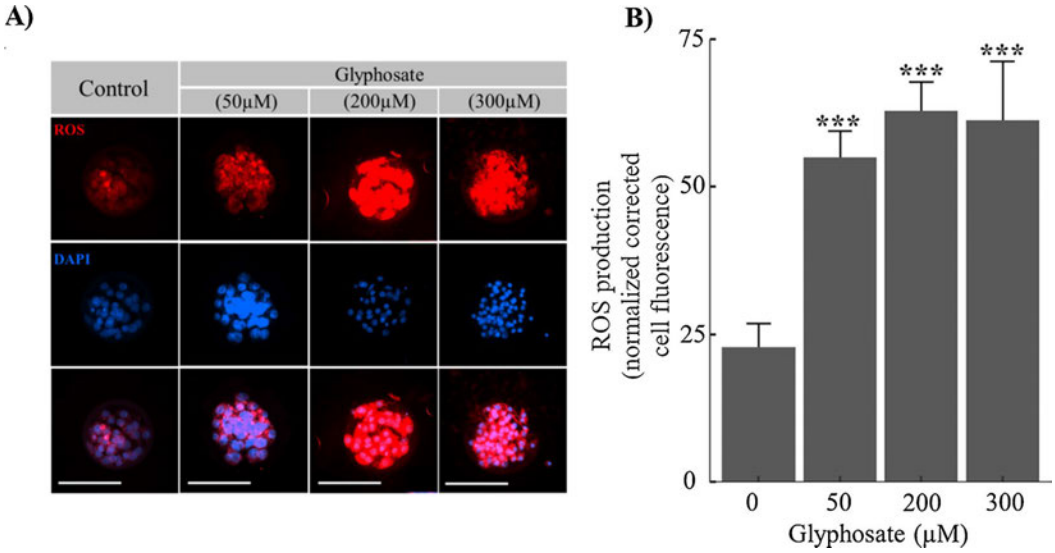
Fig. 6*: Evaluation of ROS generation in oocytes exposed to increasing glyphosate concentration
A) Representative fluorescent confocal images of metaphase II mouse oocytes stained for ROS (red, ROS Deep Red dye) and nucleus (blue, DAPI) when treated with increasing concentration of glyphosate (0, 50, 75, 100, 200 and 300 µM; some treatments omitted from figure for greater focus). Note the increase in cellular ROS as glyphosate concentration increases. Images of ROS fluorescence and DAPI were merged to assess cellular density. Scale bars: 75 µm. Images shown are from a typical experiment performed in triplicate. B) Quantification of ROS fluorescence in metaphase II mouse oocytes treated with increasing glyphosate concentration (0-300 µM). Fluorescence was quantitated in terms of corrected total cell fluorescence (CTCF), normalized for cell area, and presented as mean±SEM (Standard error of the mean). Statistical significance was indicated by * ($p<0.001$; Student's t-test). Collectively, these results display that glyphosate deteriorates oocyte quality by mediating enhancement of ROS.**



*Retrived from Yahfoufi, Z. A., *et al*., Toxicology 439 (2020) 152466

Similarly, changes in ROS production resulting from glyphosate exposure (50, 200, and 300 µM for 2 h) were also examined in embryos (Fig. 7). Glyphosate exerted a comparable effect in embryos, which showed increase in ROS production as the embryos were exposed to glyphosate. Fig. 7B shows a significant increase in ROS production in the treatment groups when compared to control ($p<0.01$ for comparisons of all treatment groups with the control).

Fig. 7*: Evaluation of ROS generation in embryos exposed to increasing glyphosate concentration
A) Representative fluorescent confocal images of mouse embryos stained for ROS (red, ROS Deep Red dye) and nuclei (blue, DAPI) when treated with increasing concentration of glyphosate (0 - 300 µM; some treatments omitted from figure for greater focus). 10 µM pyocyanin served as a positive control. Note the increase in cellular ROS as glyphosate concentration increases. Images of ROS fluorescence and DAPI were merged to assess cellular density. Scale bars: 100 µm. Images shown are from a typical experiment performed in triplicate. B) Quantification of ROS fluorescence in mouse embryos treated with increasing glyphosate concentration (0-300 µM). Fluorescence was quantitated in terms of corrected total cell fluorescence (CTCF), normalized for cell area, and presented as mean±SEM (Standard error of the mean). Statistical significance was indicated by * ($p<0.001$; Student's t-test).**



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Conclusion (by the author)

The work demonstrates for the first time that glyphosate exposure causes metaphase II oocyte quality deterioration in dual mechanisms involving i) pericentrin abnormalities, spindle fiber destruction and disappearance, and disturbance in chromosomal alignment and ii) significant interference with intracellular zinc bioavailability and ROS accumulation. The work also links ROS enhancement and zinc deficiency mediated by glyphosate exposure to possible embryo damage.

Assessment and conclusion

Assessment and conclusion by applicant:

The quality of metaphase II noncumulus oocytes and embryos from mice were investigated following glyphosate exposure at different concentrations (max 300 µM). The concentrations were in the range of those found in human blood following accidental acute exposure or suicidal attempts.

Results indicate that glyphosate provokes disruption of the microtubule organizing center and chromosomal disorganization at the mid-position of the spindle due to spindle disappearance, and defective chromosomal alignment as well as depletion of intracellular zinc bioavailability and enhancement of reactive oxygen species (ROS) accumulation in the mouse oocytes. In the embryos (not specified the source and the embryonal stage) zinc depletion and accumulation of ROS was also observed in a dose-related manner.

The article is classified as reliable with restrictions for the following reason: Not performed according to GLP or an OECD test guideline. No purity of the test substance stated. No information of the source and the embryonal stage of the embryos were provided. There were no concurrent positive control or substances known to deteriorates oocyte quality through disassembly of microtubule organizing centers (like peroxynitrite) or ROS accumulation (like hydrogen peroxide, and hypochlorous acid) or dimercapto-1-propanesulfonic acid (DMPS) for zinc depletion.

Assessment and conclusion by RMS:
The aim of the study was to investigate the effects of glyphosate (0 – 300 µM) on metaphase II mouse oocyte quality and embryo damage to obtain insight on its mechanisms of cellular action and the tolerance of oocytes and embryos towards glyphosate.

The study shows that glyphosate in a dose dependent manner and in concentrations in the range of those found in human blood following accidental acute exposure causes disruption of the microtubule organizing center and chromosomal disorganization. Also, interference with intracellular zinc bioavailability and ROS accumulation were observed in the mouse oocytes. Further, in embryos zinc depletion and accumulation of ROS was also observed in a dose-related manner.

The study is considered as supplementary data and is reliable with restrictions because of the following reasons: the purity of the test substance is unknown, solvent used for glyphosate not stated, no information of the embryonal stage of the embryos provided and no positive control included for ROS accumulation or disassembly of microtubule organizing centers.

Summary of published literature studies identified by the applicant as supplementary after detailed assessment of full-text articles (Category B)

Category B – Abou-Amer W.L. *et al.*

Data point	CA 5.6
Report author	Abou-Amer W. L. *et al*.
Report year	2010
Report title	Teratological effects induced by three pesticides in pregnant rats
Document source	Alexandria Journal of Pharmaceutical Sciences (2010), Vol. 24, No. 1, pp. 21-26
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary/reliable with restrictions (see assessment and conclusion by RMS)

Aim of the study
The aim of the study was to investigate possible teratological effects in pregnant female rats of three commonly used pesticides of which one was glyphosate. Groups of female Wistar rats were given glyphosate at 500 mg/kg bw/day by oral gavage, from the sixth day till the twentieth day of gestation. Dams body weight were recorded daily and cesarean were performed on the twentieth day of gestation.

Materials and methods

Test material:	Roundup containing 35% glyphosate E.C. provided by Pesticide Center Institute, Dokki, Cairo, Egypt
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Water
Test animals:	Rat
Strain:	Wistar
Age/weight on arrival:	Not reported/180-200 g
Source:	Helwan farm of Egyptian Organisation for Vaccine and Biological Preparations, Helwan, Cairo, Egypt
Housing:	In stainless steel cages (65 x 25 x 15 cm) with wire mesh bottom During mating: 3

	females were housed with 1 male
Acclimatisation period:	Not reported
Diet:	Pelleted diet from El-Salam Factory for Dry Ration, El-Marg, Cairo, Egypt, *ad libitum*
Water:	*ad libitum*
Environmental conditions:	Temperature: 28± 2°C

Mating procedure
Three female rats on proestrus with regular oestrous cycle were mated with one of proven fertile male. Mating was determined by the presence of a copulatory plug in the vagina or finding the sperm in the vaginal smear following overnight cohabitation. The day when spermatozoa were detected, was designated as Day 0 of gestation.

Animal assignment and treatment
Roundup was dissolved in water and a dose of 500 mg glyphosate/kg bw/day was administered by oral gavage to 10 pregnant females. A similarly constituted group of females received the vehicle and served as control. The test item was administered at a constant dosage volume of 2 mL/kg bw/day from Day 6 to Day 20 of gestation.

Maternal observations
For each pregnant female, the body weight was recorded daily from Day 6 of gestation until study termination. The animals were observed for any signs of intoxication during the experimentation period.

Cesarean section
On gestation Day 20, all females were anaesthetized, and the gravid uteri were removed, weighed and examined in situ. The number of resorption sites, implantation sites and live or dead fetuses was recorded. Live fetuses were weighed and examined for any skeletal malformation. This examination was carried out by placing the fetuses in 100% ethanol, cleared with 2% aqueous NaOH and bones stained with alizarin red to detect any skeletal abnormalities. Skeletal alterations were evaluated according to the atlas of rat skeletal anomalies.

Statistical analysis
Data were expressed as mean ± standard error of the mean (SE). Student's t-test was applied to determine the significance of differences between treated and control means.

Results

Daily oral administration of glyphosate to pregnant rats from day 6 to day 20 of gestation did not induce clinical signs of toxicity in the animals. All rats seemed to be healthy and survived the treatment.

There was a statistically significant reduction in body weight gain when compared to control animals (see Table 1). Females treated with glyphosate initially lost weight between pre-dosing and gestation day 9, but gained weight thereafter. As the time of pregnancy proceeded, the magnitude of body weight reduction of treated rats when compared to control animals decreased. On days 10, 15 and 20 of pregnancy, the reduction of body weight of rats treated with glyphosate was 95.04, 50.35 and 35.89% compared to controls (see Table 1).

Table 1*: Daily mean body weight gain of control and glyphosate treated rats from Day 6 to 20 of gestation

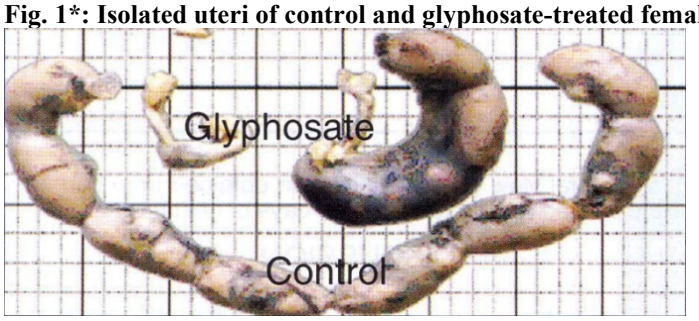
Days of Gestation	Control	Glyphosate	
	I*	I*	II**
7	6.3	-1.8	-
8	12.3	-3.6	-
9	17.5	-1.1	-
10	22.2	1.1	(95.04)
11	26.1	6.2	(76.24)
12	28.9	9.3	(67.82)
13	33.5	13.1	(60.89)
14	38.8	17	(56.18)
15	42.7	21.2	(50.35)
16	46.9	25.3	(46.05)
17	51.9	29.1	(43.93)
18	56.9	33.6	(40.94)
19	61.9	38.8	(37.31)
20	66.3	42.9	35.29

* This column includes the average gain weight (g) from the initial weight for each treatment, ** in this column II, parenthetical numbers show the percent reduction of weight of each treatment in relation to the respective control. Numbers preceded by a (-) sign indicate loss weight from the initial weight

*Retrived from Abou-Amer, W.L., *et al*., Alex. J. Pharm. Sci, Vol. 24 (1) March 2020 /21-26

At gross necropsy, the uterine horns of glyphosate-treated females appeared unequal with irregular distribution of implanted embryos and resorption sites (Fig. 1).

Fig. 1*: Isolated uteri of control and glyphosate-treated females at Day 20 of gestation



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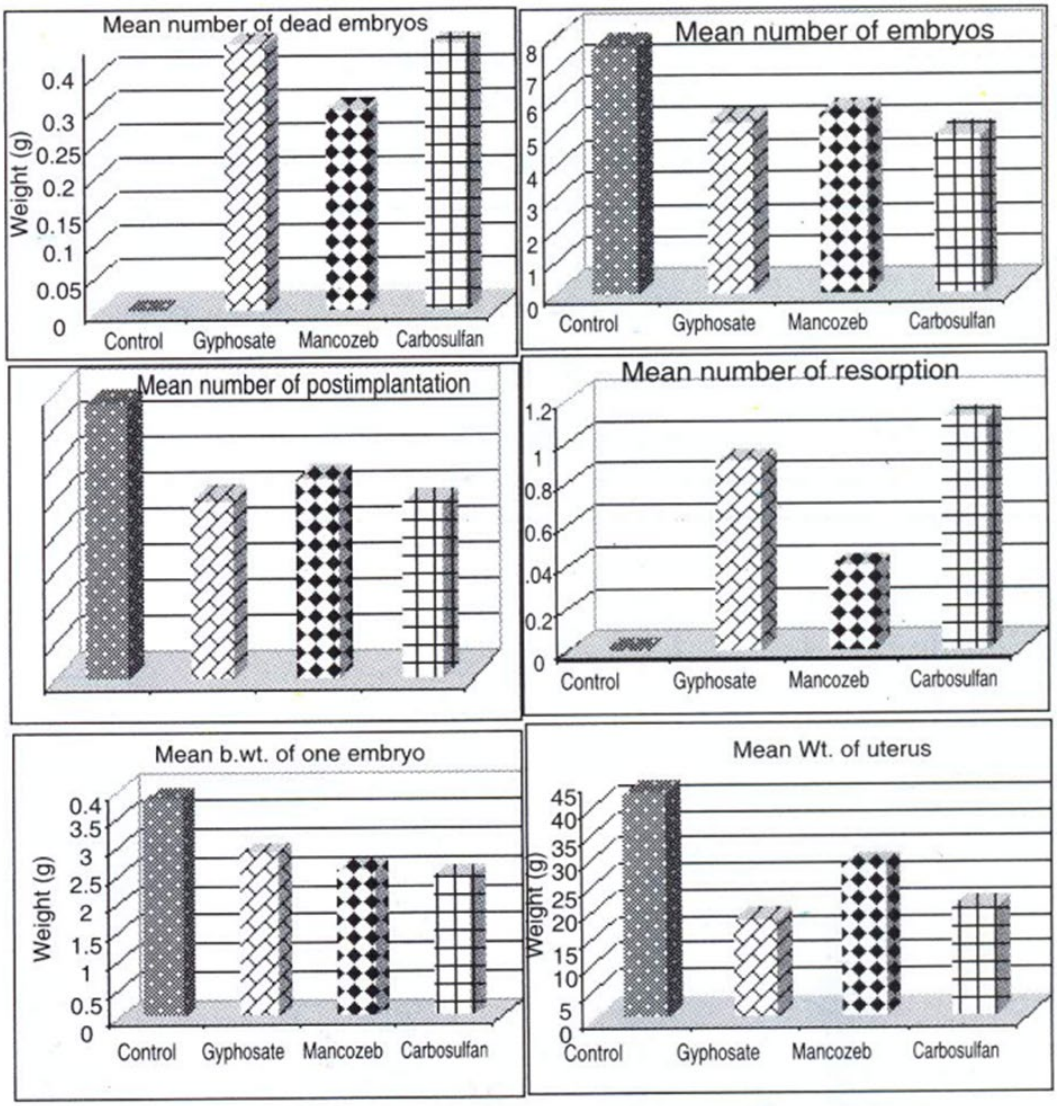
In addition, there was a statistically significant reduction in the mean number of implantation sites, the mean uterus weight and the mean number of total and live fetuses when compared to vehicle control animals (Table 2). Also the number of resorption was statistically significantly increased (Fig. 2).

Table 2*: Teratological effects observed after treatment with glyphosate

Group	Mean No. of implantation sites	Mean No. of resorption sites	Mean weight of uteri (g)	Mean No. of fetuses		Mean weight of one fetus (g)
				Total	live	
Control	7.7 ± 1.25	0	42.56 ± 1.01	7.7 ± 1.25	7.7 ± 1.25	3.87 ± 0.07g
Glyphosate	5 ± 0.57*	0.9 ± 0.51	18.44 ± 4.15*	4.1 ± 0.92*	3.7 ± 2.89*	2.89 ± 0.3g*

*Retrived from Abou-Amer, W.L., *et al*., Alex. J. Pharm. Sci, Vol. 24 (1) March 2020 /21-26

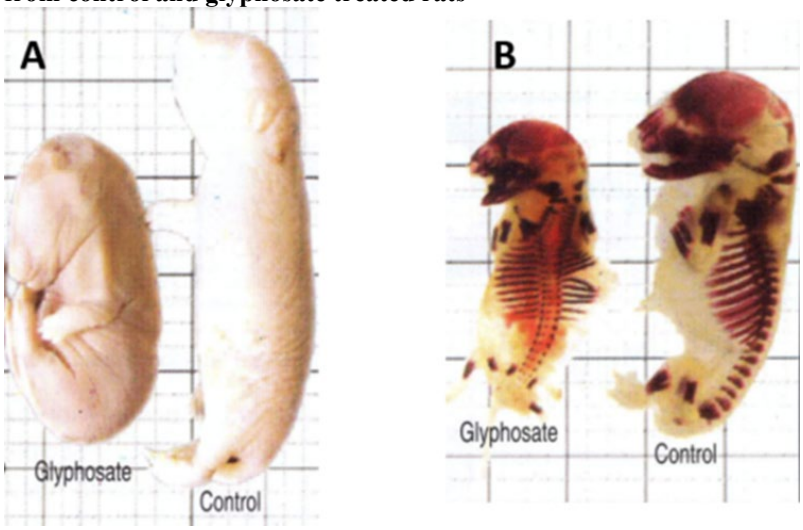
Fig. 2*: Teratogenic parameters of rats treated with glyphosate



*Retrived from Abou-Amer, W.L., *et al*., Alex. J. Pharm. Sci, Vol. 24 (1) March 2020 /21-26

Glyphosate caused significant loss in fetal weight when compared with controls. This may be due to the toxicity of the pesticide itself or may be explained by the decreased food and water intake and the stress induced by exposure to repeated doses. Growth retardation commonly occurred among fetuses of dams treated with toxicants. Fetal examination revealed a statistically significant loss in fetal size and weight (Fig. 3A) and skeletal malformations (Fig. 3B). In the treated group, effects were represented by less ossification of most parts of skull and legs, as well as complete loss of ossification in the digits and caudal vertebrae in comparison with those of control.

Fig. 3*: Fetus size obtained from uteri (A) and lateral view of fetus skeleton (B) after 20 days of gestation from control and glyphosate treated rats



*Retrived from Abou-Amer, W.L., *et al*., Alex. J. Pharm. Sci, Vol. 24 (1) March 2020 /21-26

Conclusion

In summary, the investigations have shown that glyphosate causes significant reductions in body weight gain (see Table 1) of dams and different teratological effects when compared to control animals. Besides irregular distribution in implantation sites, increased resorption, number of dead fetuses and fetal weights were decreased and skeletal malformation was observed.

Assessment and conclusion

Assessment and conclusion by applicant:

5.4.1 case b) Relevant but supplementary information: Study is done with pesticide formulations with only one dose per pesticide treatment group established. The study contains insufficient data, therefore supplementary only.

The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each to the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components. The uncertainty associated with whether the product contains polyethoxylated tallow amine (also polyoxyethyleneamine, POEA) or not, suggests that the findings in this paper should be treated with high level of caution.

Further points of clarification:

In the present publication, glyphosate was found to induce teratogenic effects when administered to pregnant rats. Besides a reduced body weight gain in the dams during pregnancy, the number of implantation sites was reduced and more fetuses were resorbed during pregnancy. The number of live fetuses and their weights was reduced when compared to control animals and growth retardation and malformation of fetal skeletons were observed.

Limited information on the test material was provided (CAS, batch and purity were not reported). The test item was used as a formulation and there was no information on the other ingredients within the formulation. Historical control data on reproductive parameters were not given.

The study is assigned to section 5.6.2 and considered relevant but to provide supplementary information (case B), as it was performed with one glyphosate dose of one formulation only. There was no sufficient characterisation of the test material, as the batch and purity were not reported. Glyphosate was used as Roundup formulation and no information was given on other ingredients. In addition, no historical control data were

reported on reproductive parameters. Therefore, the publication was considered supplementary only.

Assessment and conclusion by RMS:
In this study a dose of 500 mg glyphosate/kg bw/day was administered by oral gavage to 10 pregnant females from day 6 to day 20 of gestation. A similarly constituted group of 10 females received the vehicle and served as control.

Treatment with glyphosate did not induce clinical signs of toxicity in the animals and all rats seemed to be healthy and survived the treatment. A reduced body weight gain in the dams during pregnancy was observed when compared to control animals. Further, the mean number of implantation sites, the mean uterus weight and the mean number of total and live fetuses were reduced when compared to control animals. Also the number of resorption was increased. Fetal examination revealed a loss in fetal size, weight and skeletal malformations in the glyphosate treated animals when compared to controls. Glyphosate treatment also caused less ossification of most parts of skull and legs, as well as complete loss of ossification in the digits and caudal vertebrae in comparison with those of control.

The study is considered as supplementary data only. The study is carried out with a formulation of glyphosate, thus effects caused by co-formulants cannot be excluded. The study is reliable with restrictions because of the following reasons: the test substance is not sufficiently characterised (particularly, purity and batch not specified), only one dose was used, low number of animals, acclimatisation period not reported, temperature exceeded limit, food consumption not measured, individual data missing, necropsy of dams not preformed, assessment of AGD, T4, T3 and TSH in dams not performed, sex ratio missing, no historical control data and positive control missing.

Category B – de Almeida L.L. *et al.*

Data point	CA 5.6
Report author	de Almeida L. L. *et al*.
Report year	2017
Report title	Effects of melatonin in rats in the initial third stage of pregnancy exposed to sub lethal doses of herbicides
Document source	Acta Histochemica (2017), Vol. 119, No. 3, pp. 220-227
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary/ reliable with restrictions (see assessment and conclusion by RMS)

Aim of the study
The aim of the study was to evaluate the possible changes in reproductive and hepatic parameters following exposure to Glyphosate-Roundup and Paraquat as well as its prevention by simultaneous application of melatonin.

Groups of 5 female rats received either 500 mg/kg bw Glyphosate-Roundup, or 500 mg/kg bw Glyphosate-Roundup plus simultaneous treatment with 10 mg/kg bw melatonin, or 500 mg/kg bw/day Glyphosate-Roundup plus simultaneous treatment with 50 mg/kg bw Paraquat, or 500 mg/kg bw/day Glyphosate-Roundup plus simultaneous treatment with 10 mg/kg bw/day melatonin and 50 mg/kg bw/day Paraquat during day 1 to 7 of gestation by oral gavage.

On day 7 of pregnancy, the rats were anaesthetized and euthanized, followed by laparotomy to remove their reproductive tissues and liver. Body and ovary weights were taken and the number of implantation sites, corpora lutea, preimplantation losses, implantation rates were counted and histopathology of the implantation sites,

morphometry of the surface and glandular epithelia of endometrium were investigated. Samples of liver tissue were collected for analysis of the oxidative stress parameters, lipid peroxidation and glutathione.

Materials and methods

Test material:	Roundup made of 360 g/L glyphosate (N phosphonomethyl glycine) and 16% (w/v) polyoxoethylene amine, Sigma Aldrich, St. Louis, Missouri, USA
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Physiological saline (0.9% solution)
Test animals:	Rat
Strain:	Wistar
Age/weight on arrival:	90 days/200 ± 20 g
Source:	Laboratory of Histology of the Department of Animal Morphology and Physiology, Research Center (Cenapesq), Federal Rural University of Pernambuco, Brazil
Housing:	In controlled cages. During mating, one male was maintained in a cage with 2 females.
Acclimatisation period:	10 days
Diet:	*ad libitum*
Water:	*ad libitum*
Environmental conditions:	Temperature: 22± 2°C Light/dark cycle: 12 hr light 12 hr dark Humidity: 60 ± 10%

Mating procedure and verification of copula
Two female rats with 3 regular oestrous cycles were mated with one male until mating was verified. After mating, a vaginal smear was made every day. The presence of a vaginal plug or sperm cells in the vaginal smear was taken as an indication of effective copulation. The collection of vaginal smears was performed using swabs of sterile cotton (Absorve) and subsequent deposit of biological material in histological slides, which were stained with Harris Shorr method, then the slides were preserved under glass coverslips using Entellan mounting medium (EMS, Hatfield, PA, USA). All stained slides were examined using a Leica DM500 light microscope (Leica, Wetzlar, Germany).

Animal assignment and treatment
Glyphosate-Roundup was dissolved in physiological saline (0.9%) and a dose of 500 mg/kg bw/day was administered by oral gavage between 9-10 am to 5 pregnant females during day 1-7 of pregnancy while, melatonin 10 mg/kg bw, diluted in an ethanol/saline 4% solution was administered intraperitoneally at 6 pm during day 1-7 of pregnancy. Similarly constituted groups of rats received 500 mg/kg bw/day Glyphosate-Roundup plus 10 mg/kg bw/day melatonin, 500 mg/kg bw/day Glyphosate-Roundup plus 50 mg/kg bw Paraquat or 500 mg/kg bw/day Glyphosate-Roundup plus 10 mg/kg bw/day melatonin plus 50 mg/kg bw/day Paraquat during day 1-7 of gestation. Additional animals received either 50 mg/kg bw/day Paraquat or 50 mg/kg bw/day Paraquat plus 10 mg/kg bw/day melatonin.

Maternal observations and experimental procedure
Body weight and survival of the pregnant females were monitored daily. At scheduled Caesarean section on gestation day 7, the rats were immediately euthanized with thiopental (40 mg/kg), followed by laparotomy to remove the uterine horns with the implantation sites and the ovaries. Histopathological analysis of the implantation sites was performed, coupled to morphometry of the surface and glandular epithelia of the endometrium. The weight of the female rats, ovaries, number of implantation sites, corpus luteum, implantation rate and preimplantation loss were calculated.

Measurement of lipid peroxidation (LPO)
Oxidative stress analysis was performed by measuring the levels of TBARS (Thiobarbituric Acid Reactive Substance). Samples of the liver were ground in potassium chloride (KCl) 1.15% at a proportion of 10 mL/g until the complete homogenization of the material. The homogenate was then transferred to a test tube and 2 mL of the reagent (0.375% thiobarbituric acid and 75 trichloroacetic acid) were added for each mL of the mixture. Duplicate tubes were sealed and heated in a warm bath at 100◦C for 15 min. The supernatant was separated and absorbance was measured at 535 nm. All doses were performed in triplicate.

Measurement of glutathione (GSH) concentration
The levels of reduced glutathione (GSH) were quantified by the concentration of non-protein sulfhydryl groups in the homogenate. The other half of each homogenate was mixed with an equal volume of 10% (w/v) of trichloroacetic acid (TCA) to precipitate the proteins and then centrifuged at 1000 x g for 10 min. Further, 1 mL of the supernatant was mixed with 1.5 mL of a reaction medium with Tris at 100 mM (pH 8.9) and 0.4 mM of 5.5-dithiobis-2-nitrobenzoic acid. The samples were incubated at room temperature for 5 minutes and absorbance measured at 412 nm. Results were compared with a standard curve by α cysteine and corrected for the protein rate of the initial homogenates. All doses were tested in triplicate.

Histopathological examination of the implantation sites
After laparotomy and the removal of the uterine horns, the implantation sites and the corpora lutea were counted with a stereomicroscope Olympus SZ40 and pre-implantation losses and implantation rates were calculated. Implantation sites were immersed in buffered formaldehyde 10% and fixed for 24 h. After the fixture, the tissues were washed in a PBS solution, dehydrated in a series of increasing alcohol concentrations (80, 90, 95 and 2 × 100%) and placed in methacrylate historesin glycol (Historesin Leica). Further, 4 m cuts, stretched in the water and placed on the laminas, were obtained by microtome Leica RM 2245 with glass razor. They were dried in a buffer at 60 ºC for 1 min and underwent staining with Haematoxylin Eosin (H.E.) To select the fields to be analysed, was adopted the following criteria: only the fields with the blastocyst presence in deployment process for histopathology. The slides were analysed under a light microscope Leica DM500 in duplicate/animal and photographed by camera Leica EC3 locked to a microscope for histopathological.

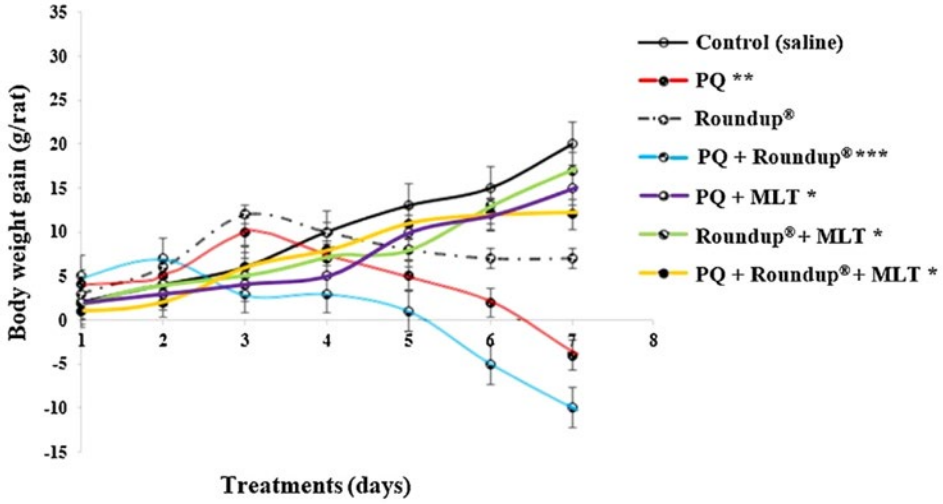
Morphometrical analysis
Morphometric analysis comprised images (2 slides/animal/group) with camera Leica EC3 coupled to a microscope, and the parameters: height of surface and glandular epitheliums of the endometrium and endometrial glands diameter were analysed by Leica Application Suite (LAS) EZ. Ten measurements/field of the parameters under analysis were undertaken for each animal/group. To select the fields to be morphometrically analysed, the following criteria were adopted: select and capture fields on the perimeter along the decidualization zone in each histological sample (slide), i.e. in the area where there are the luminal epithelium and endometrial glands.

Statistical analysis
Rates were given as averages ± standard error of average. Differences between groups were determined by the analysis of variance (one way ANOVA), followed by Tukey's test when a difference was detected. Significance level for rejection of null hypothesis was fixed at 5% ($p < 0.05$).

Results

Weight of pregnant rats
There was a decrease in body weight gain in rats treated with the herbicides Glyphosate-Roundup and Paraquat and simultaneously with melatonin. After individual treatment with Glyphosate-Roundup, there was no body weight gain from day 3 of gestation onwards when compared to control animals. After treatment with Glyphosate-Roundup plus Paraquat there was a statistically significant decrease in body weight.

Fig. 1*: Body weight gain during exposure to Glyphosate-Roundup and Paraquat (PQ) and treatment with melatonin (MLT). All rates are given as means ± SE of seven experimental groups



*Retrived from de Almeida, L.L., *et al*., Acta Histochemica 119 (2017) 220-227

A reduction in ovary weight and a decrease in the number of implantation sites and implantation rates were observed in rats exposed to Glyphosate-Roundup and Paraquat both when administered individually and simultaneously. Weight of ovaries among groups treated with melatonin was similar to the control animals. The same result occurred for the number of implantation sites and the implantation rate.

Table 1*: Reproductive performance parameters (mean ± SE) after exposure to Glyphosate-Roundup, Paraquat (PQ), and melatonin (MLT)

[1]Data represent the mean ± SE of the reproduction performance of experimental groups exposed to herbicides and MLT.

Parameters	[2]Treatments							
	Control	PQ	Roundup®	Roundup® + PQ	PQ + MLT	Roundup® + MLT	Roundup® + PQ + MLT	F Statistic P
Number of pregnant rats	5	5	5	5	5	5	5	
Weight of ovaries (mg)	63,2 ± 0,5a	35,4 ± 1,9c	45,0 ± 2,3b	30,6 ± 1,0d	56,0 ± 2,0a	54,4 ± 1,7a	54,6 ± 1,7a	$75{,}45^{0{,}0001}$
Number of implantations	12,4 ± 0,5a	5,4 ± 0,4b	7,2 ± 0,6b	2,6 ± 0,4c	11,0 ± 0,7a	11,8 ± 0,7a	9,8 ± 0,6a	$41{,}51^{0{,}0001}$
[3]Implantation rate (%)	83,6 ± 2,0a	49,0 ± 2,2b	58,2 ± 1,3b	29,2 ± 4,4d	75,8 ± 3,7a	78,2 ± 4,2a	73,7 ± 2,6a	$41{,}29^{0{,}0001}$
Number of corpora lutea	15,2 ± 0,6a	8,8 ± 0,5b	9,4 ± 0,9b	5,8 ± 0,6c	13,2 ± 0,4a	14,0 ± 0,7a	13,0 ± 0,8a	$27{,}61^{0{,}0001}$
[4]Pre-implantation losses (%)	16,4 ± 1,5a	40,6 ± 1,0b	35,5 ± 1,2b	55,5 ± 3,1c	23,4 ± 3,6a	18,3 ± 1,3a	22,9 ± 3,0a	$37{,}66^{0{,}0001}$

[1] Means followed by different letters are significantly different by Tukey HSD ís test at 5%.
[2] Roundup (glyphosate-Roundup® 500 mg/kg/day), PQ (Paraquat 50 mg/kg/day) and MLT (Melatonin 10 mg/kg/day).
[3] (Total number of implantations/number of corpora lutea) × 100.
[4] (Number of corpora lutea – number of implantations/number of corpora lutea) × 100.

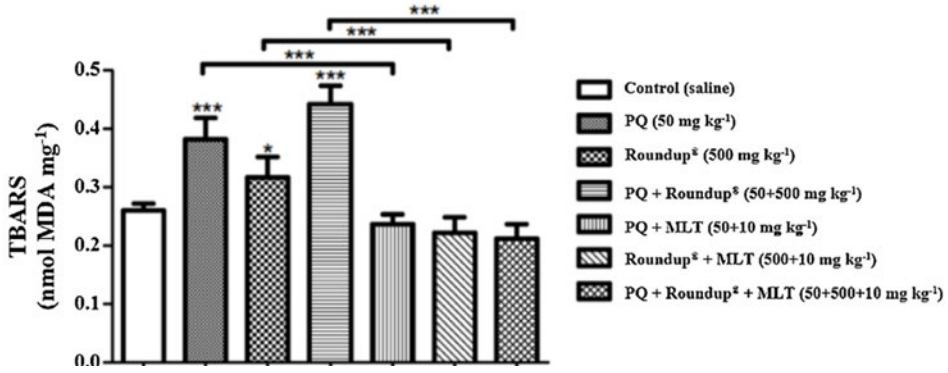
*Retrived from de Almeida, L.L., *et al*., Acta Histochemica 119 (2017) 220-227

Microscopical analysis of the ovaries showed that individual and simulatneous exposure of Glyphosate-Roundup and Paraquat also reduced the total number of corpora lutea and increased the percentage of pre-implantation losses when compared to the control group. The number of corpora lutea and pre-implantation losses of melatonin treated animals were comparable to those of control animals.

Measurement of lipid peroxidation (LPO)

There was a statistically significant increase in TBARS levels in the liver tissue after individual or simultaneous exposure to Glyphosate-Roundup and Paraquat when compared to the control animals. Simultaneous exposure of Glyphosate-Roundup and Paraquat caused the highest rates in TBARS levels. There was no induced lipid peroxidation after melatonin treatment.

Fig. 2*: Measurement of lipid peroxidation (means ± SE of hepatic TBARS) after treatment with herbicides. *Indicates significant difference between control and exposed group *p < 0.05 ***p < 0.001 and recovery when treated with melatonin (MLT) ***p < 0.001 (ANOVA/Tukey)



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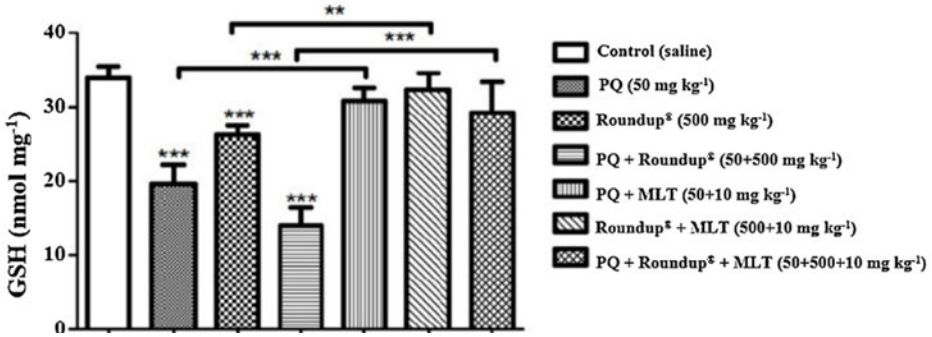
Measurement of glutathione (GSH) concentration

There was a statistically significant decrease in glutathione levels after individual and simulateous exposure of Glyphosate-Roundup and Paraquat when compared to control animals. Groups treated with melatonin had similar rates to those of control animals.

Fig. 3*: Measurement of glutathione (GSH) concentration (mean ± SE) after treatment with herbicides. *Indicates significant difference between control and exposed group ***p < 0.001 and recovery when treated with melatonin (MLT) ***p < 0.001 ** p < 0.01 (ANOVA/Tukey)



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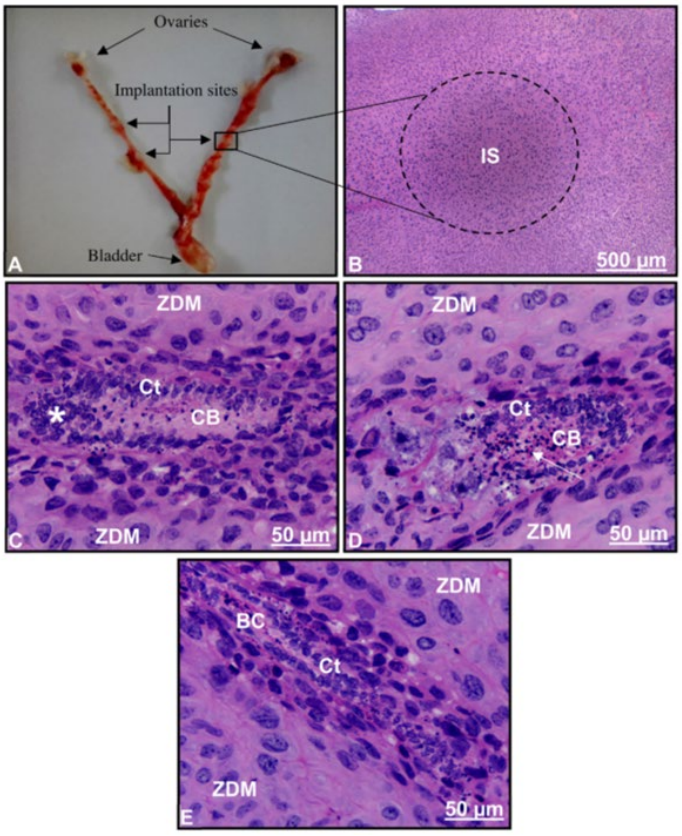
Histopathological examination of implantation sites

Following treatment with Glyphosate-Roundup and Paraquat simultaneously there was an observed disorganization of the cytotrophoblast and cell degeneration within the blastocyte cavity. Implantation sites in rats treated with melatonin had a structural organization similar to that in rats of the control group.

Fig. 4*: Photographs and photomicrographs of representative uteri collected and implantation sites on the 7th day post coitum of pregnant rats exposed to herbicides Paraquat and Glyphosate-Roundup and treated with melatonin. A: Uterine horns with implantation sites. B: Overview on the implantation site (IS). C: Control group with preserved structures such as cytotrophoblast (Ct), embryoblast (asterisk), blastocystic cavity (Bc) and a well defined mature deciduality zone (ZDM). D: Treatment with Glyphosate-Roundup, Paraquat and melatonin simultaneously; disorder of blastocyst (Bc) and cell degeneration (arrow) of cytotrophoblast within the blastocystic cavity. E: Treatment with melatonin; levels of structural organizations similar to those of female rats in control group.



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Morphometrical analysis

Morphometric analysis of the surface and glandular epithelium of the endometrium glands revealed a decrease in diameter of endometrium glands after both individual and simultaneous exposure of Glyphosate-Roundup and Paraquat when compared to control animals. The investigated parameters after melatonin treatment were similar to those of control animals.

Fig. 5*: Morphometric analysis of endometrium (mean ± SE) after treatment with herbicides

[1]Data represent the mean ± SE of morphometric analysis of endometrium of experimental groups exposed to herbicides and MLT.

Parameters	[2]Treatments							
	Control	PQ	Roundup®	Roundup® + PQ	PQ + MLT	Roundup® + MLT	Roundup® + PQ + MLT	F Statistic P
Number of pregnant rats	5	5	5	5	5	5	5	
Height of surface epithelium (µm)	23,7 ± 0,5a	13,9 ± 0,7c	18,2 ± 0,4b	9,9 ± 0,6d	21,8 ± 0,7a	22,2 ± 0,4a	21,2 ± 0,7a	$64{,}20^{0.0001}$
Heights of glandular epithelium (µm)	21,9 ± 0,7a	13,1 ± 0,4b	16,1 ± 0,7c	9,8 ± 0,7d	20,4 ± 0,4a	21,3 ± 0,4a	19,2 ± 0,4a	$54{,}17^{0.0001}$
Diameter of endometrium glands (µm)	37,1 ± 1,5a	20,0 ± 0,6c	26,9 ± 1,3b	14,0 ± 0,7d	36,0 ± 1,5a	37,2 ± 1,0a	33,8 ± 0,9a	$49{,}57^{0.0001}$

[1] Means followed by different letters differ significantly by Tukey HSDís test at 5%.
[2] Roundup® (500 mg/kg/day), PQ (Paraquat 50 mg/kg/day) and MLT (Melatonin 10 mg/kg/day).

*Retrived from de Almeida, L.L., *et al*., Acta Histochemica 119 (2017) 220-227

Conclusion

In conclusion, treatment with Glyphosate-Roundup individually or simultaneously with Paraquat strongly negatively affected body weight gain during the first week of pregnancy. In addition, there was a statistically significant reduction in ovary weight and in the number of implantation sites, as well as a reduction in the total

number of corpora lutea and a significant increase in the percentage of pre implantation losses when compared to those of control. The changes in reproductive parameters were not observed when the animals were simultaneously treated with melatonin.

At histopathological examination of the implantation sites, a disorder of the cytotrophoblast and cell degeneration within the blastocyst cavity was noted in animals simultaneously treated with Glyphosate-Roundup and Paraquat. Morphometry revealed a reduction in surface and glandular epithelia and in the diameter of the endometrial glands after both individual and simultaneous exposure of Glyphosate-Roundup and Paraquat when compared to control animals. The investigated parameters after melatonin treatment were similar to those of control animals.

In the liver of animals exposed to Glyphosate-Roundup and Paraquat individually or simultaneously, serum levels of TBARS were found to be significantly elevated while serum level of reduced glutathione (GSH) was significantly lowered. However, treatments with melatonin exhibited improvements in reproductive parameters, as well as reduced lesions in the implantation sites. It was concluded that melatonin treatment acts efficiently against toxic effects on the reproductive system and the embryo, as well as in the liver. Also, melatonin improves fertility indexes such as the prevention of the blastocytes' morphological integrity, gain in maternal body weight, ovary weight, number of corpora lutea, and viability of the embryo implantation.

Assessment and conclusion

Assessment and conclusion by applicant:
5.4.1 case b) Relevant but supplementary information: Formulation tested at high doses of 500 mg/kg bw/day (Roundup), therefore supplementary only.

The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains polyoxyethylene tallow amine (POEA), which is not permitted for use in formulated herbicidal products in the EU.

Further points for clarification:
The objective of the present study was the investigation of reproductive effects and the induction of oxidative stress in the liver in pregnant rats after treatment with the glyphosate formulation Roundup, as individual substance, associated with the herbicide Paraquat and in combination with melatonin.

Roundup formulation alone and in association with Paraquat significantly impaired the dams' body weight development and caused major changes in reproductive parameters. The changes in reproductive parameters were not observed when the animals were simultaneously treated with melatonin. Histopathological examination of the implantation sites revealed a disorder of the cytotrophoblast and cell degeneration within the blastocyst cavity in animals simultaneously treated with Roundup, Paraquat, and melatonin.

Morphometry revealed a reduction in surface and glandular epithelia and in the diameter of the endometrial glands. Further, lipid oxidation and reduction in glutathione indicated oxidative stress in herbicide treated animals.

There are several deficiencies in the reporting of methods and results, which adversely affect the reliability of the data and results reported. Only one dose level of 500 mg/kg bw/day was tested, which represents a high dose level of glyphosate, which does not correspond to physiological levels.

There was no information on the test material purity, batch and composition reported. Historical control data on reproductive parameters or oxidative stress in the liver were not given.

Further, it is unclear if the control animals remained untreated or received the vehicle (0.9% saline). In addition, no historical control data were provided on reproductive parameters in the animals.
Effects of the glyphosate formulation Roundup were investigated after treatment with Roundup alone, after treatment in association with Paraquat and in association with melatonin. Although significant differences for reproductive parameters in herbicide and melatonin associated herbicide treated animals were shown, histopathological examination of implantation sites were only provided for animals treated with Roundup, Paraquat and melatonin simultaneously.

Assessment and conclusion by RMS:
The aim of the study was to investigate reproductive effects and the induction of oxidative stress in the liver by simultaneous application of 10 mg/kg bw/day melatonin to pregnant female rats exposed to 500 mg/kg bw Glyphosate-Roundup and 50 mg/kg bw/day Paraquat during day 1 to 7 of gestation.

Exposure of Glyphosate-Roundup formulation alone and in association with Paraquat significantly impaired the dams' body weight development and caused changes in reproductive parameters. These changes were not observed when the animals were simultaneously treated with melatonin.

The study is considered as supplementary data only. The study is carried out with a formulation of glyphosate containing polyethylene tallow amine, thus effects caused by co-formulants cannot be excluded. The study is reliable with restrictions because of the following reasons: the test substance is not sufficiently characterised (particularly, purity and batch not specified), only day up to day 7 of gestation studied, small group sizes of animals used, only one dose used, not clear if controls were administered vehicle, no details on food or food consumption, individual data missing, histopathological examination of implantation sites only provided for animals treated with Glyphosate-Roundup, Paraquat and melatonin simultaneously, clinical observations and historical control data not presented.

Category B – Cai W. *et al.*

Data point	CA 5.6
Report author	Cai W. *et al*.
Report year	2020
Report title	Low-dose Roundup induces developmental toxicity in bovine preimplantation embryos *in vitro*.
Document source	Environmental science and pollution research international, (2020) Vol. 27, No. 14, pp. 16451-16459
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	Not previously submitted
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Supplementary/ reliable with restrictions (see assessment and conclusion by RMS)

Aim of the study
The aim of this study was to investigate the effects of Roundup on the bovine preimplantation embryo.

Materials and methods

Test material:	Roundup from Monsanto Co., St. Louis, MO, USA purchased from a commercial source containing 360 g/L of glyphosate
Lot/Batch:	Not reported
Purity:	Not reported
Vehicle:	Synthetic oviductal fluid medium supplemented with amino acids (SOFaa)
Bovine *in vitro*:	*In vitro* fertilized oocytes

Collection and in vitro maturation of bovine oocytes
Bovine ovaries were obtained from a local abattoir and transported to the laboratory within 2 h after the slaughter in sterile saline at 20 to 22°C in a thermos bottle. Cumulus oocytes complexes (COCs) were aspired from 2 to 8 mm follicles using an 18-gauge needle into PBS plus 0.5 IU/mL heparin and 5% FBS. COCs with even cytoplasm and surrounded by compact cumulus cells were collected from the follicular fluid and PBS mixture. The collected COCs were washed twice and then incubated in TCM-199 supplemented with 10% FBS,

1 μg/mL 17β-estradiol and 0.075 IU/ mL human menopausal gonadotropin for 20 h at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization
The straws of frozen semen from an elite bull were purchased from the Cattle Reproduction and Breeding Centre of Liaoning Province (Liaoning, China). The frozen semen was thawed in 35°C water for 30 s, and motile spermatozoa were separated by using Percoll density gradients (45% and 90%). For IVF, mature COCs were incubated with 1 × 106 spermatozoa/mL in Fert-Talp medium supplemented with 20 μg/mL heparin, 1.65 μg/mL hypotaurine, 0.27 μg/mL epinephrine, and 4.5 μg/mL penicillamine at 38.5°C in 5% CO_2 in humidified air for 20 h. After IVF, presumptive zygotes were treated in 1 mg/mL hyaluronidase in PBS, and the attached spermatozoa and cumulus cells were removed by repeated pipetting. Presumptive zygotes were cultured in the synthetic oviductal fluid medium (SOF) supplemented with essential and nonessential amino acids and 8 mg/mL bovine serum albumin (BSA) (SOFaa) at 38.5°C under a humidified atmosphere of 5% CO_2 in air. After 24 h of cultivation, only the cleaved 2-cell embryos were selected for the following experiments.

In vitro embryo culture and Roundup dosage
Every 8 to 12 selected 2-cell embryos were cultured in a 50-μL droplet of SOFaa or SOFaa supplemented with different concentrations of Roundup for 144 h during which one-half of the medium was replaced at 72 h. The development was monitored every 24 h. First a dose-ranging assessment performed with 0.01~2% Roundup (36~7200 ppm) in SOFaa. If such doses were too toxic, we further determined the suitable dosage by testing concentrations of Roundup below 36 ppm (0.01%). After the dosage range was determined, we examined the *in vitro* embryo development in SOFaa containing different concentrations of Roundup based on the selected range.

Calcium detection
The calcium levels in bovine embryos were detected with Fluo3-AM. The embryos were incubated in 2 μM of Fluo3-AM made up in SOFaa containing 0.01% pluronic F127 for 30 min. Then, the embryos were washed 3 times in PBS. Immediately, they were detected at 488 nm under a Nikon A1+ laser scanning confocal microscope. The intracellular calcium intensity was analysed by using ImageJ (× 1.38) software. The image background was subtracted, and then the pixel value of fluorescence was measured using the region of interest (ROI) function.

Reactive oxygen species assay
The intracellular oxidative stress in bovine embryos was detected by using Reactive Oxygen Species Assay Kit. The embryos were incubated in SOFaa containing 4 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min. Then they were washed 3 times in PBS. Immediately, the ROS level in the individual embryo was detected at 488 nm under an A1+ laser confocal microscope. For quantitative analysis of ROS levels in the embryos, fluorescence images were analysed by using ImageJ. The image background was subtracted, and then the pixel value of fluorescence was measured using the ROI function.

Differential cell counting and apoptosis detection
The TUNEL assay and CDX2 immunofluorescence were combined to simultaneously detect apoptotic cells and inner cell mass/trophectoderm (ICM/TE) cell numbers in bovine blastocysts. The apoptotic nuclei were labelled by using a DeadEnd Fluorometric TUNEL System. All manipulations were done at room temperature unless otherwise stated. Between two steps, the blastocysts were washed three times in PBS plus 1 mg/mL polyvinylpyrrolidone-40 (PBS/PVP) for 5 min, unless otherwise stated. The blastocysts were fixed in 4% paraformaldehyde in PBS for 20 min and permeabilized in 0.2% Triton X-100 in PBS for 5 min. The blastocysts were equilibrated in equilibration buffer for 8 min and then incubated with FITC-conjugated dUTP and terminal deoxynucleotidyl transferase in equilibration buffer at 37°C for 60 min in the dark. The tailing reaction was terminated in 2× SSC for 15 min. Whereafter, the blastocysts were blocked in 0.3% BSA in PBS (blocking solution) for 1 h. Then they were immediately incubated in 1:100 dilutions of mouse anti-CDX2 antibody in blocking solution overnight at 4°C. Antibodies that bound to blastocysts were probed with TRITC-conjugated donkey antimouse secondary antibody (1:400 dilutions) in blocking solution for 20 min. The nuclei were counterstained with DAPI. The blastocysts were observed under an A1+ laser confocal microscope. The total nuclei were identified by their blue (pseudocolor) fluorescence at 405 nm. TE cells were identified by the red (pseudocolor) fluorescence in their nuclei at 561 nm. The apoptotic cells were recognized by the green (pseudocolor) fluorescence at 488 nm. Apoptosis index indicated the incidence of apoptotic cells in blastocysts and was calculated via the formula: (apoptotic cell number/total cell number) × 100.

Statistical analysis
Each experiment was independently performed at least three times. The data were analysed using SigmaStat 3.5 software. The comparisons were performed with oneway ANOVA followed by a Holm-Sidak test. Values of $P < 0.05$ were considered significantly different.

Results

The *in vitro* development of the 2-cell embryos cultured in SOFaa supplemented with 0.01~2% Roundup were examined. As shown in Table 1, the embryos died quickly in 2% Roundup in SOFaa. When cultured with 0.2% Roundup, embryos were blocked at the 2-cell to 4-cell stage and underwent degradation. Even if we decreased Roundup concentrations to 0.02% or 0.01%, the embryo development was still arrested before the compaction stage. The results indicate that the doses of 0.01~2% Roundup are too toxic to bovine embryos.

Table 1*: Effects of 0.01~2% Roundup on *in vitro* development of bovine preimplantation embryos

Roundup	No. of 2-cell embryos	No. (%) of embryos developed to		
		4-cell	8-cell	Compaction
No Roundup	30	26 (86.7 ± 6.7)[a]	19 (63.3 ± 3.3)[a]	17 (56.7 ± 6.7)
0.01% (36 ppm)	36	4 (11.1 ± 2.8)[b]	2 (5.6 ± 2.8)[b]	0 (0)
0.02% (72 ppm)	36	3 (8.3 ± 4.8)[b]	2 (5.6 ± 5.6)[b]	0 (0)
0.2% (720 ppm)	36 *	2 (5.6 ± 5.6)[b]	0 (0)[b]	0 (0)
2% (7200 ppm)	33 §	0 (0)[b]	0 (0)[b]	0 (0)

After 24 h of IVF, the cleaved 2-cell embryos were cultured in SOFaa supplemented with no Roundup (control), 0.01%, 0.02%, 0.2%, and 2% of Roundup. The experiment was repeated three times. The percentage of developmental rate was presented as mean ± SEM

[a, b] Within the same column, values with different superscripts were significantly different, $P < 0.001$

*The embryos were blocked at the 2-cell to 4-cell stage and died in 24 h of Roundup incubation

§ The 2-cell embryos died in 1 h of Roundup incubation

*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

Determination of the rough range of Roundup concentrations suitable for bovine embryo toxicological analysis
The rough range was determined by monitoring the *in vitro* development of the 2-cell embryos cultured in SOFaa containing wide-spectra concentrations of Roundup. When cultured with 7.2 ppm Roundup (containing 7.2 mg/L glyphosate), embryos could survive beyond the compaction stage. Therefore, we cultured 2-cell embryos with 7.2, 3.6, 0.36, and 0.036 ppm Roundup. The embryos survived to compacted embryos at similar developmental rates. However, there was a notable difference in the blastocyst developmental rates among these groups. As shown in Table 2, no or few blastocysts formed in the 7.2 or 3.6 ppm Roundup group. When the concentration of Roundup decreased to 0.36 or 0.036 ppm, the blastocyst development was improved significantly. And the blastocyst rates in these two groups were comparable with that in the control group (no Roundup). Based on the results, we next chose the doses between 3.6 and 0.36 ppm Roundup for further analysis.

Table 2*: The preliminary assessment of Roundup concentrations suitable for analysing its toxicity on bovine embryos

Roundup	No. of 2-cell embryos	No. (%) of embryos developed to	
		Compaction	Blastocyst
No Roundup	57	34 (59.6 ± 1.5)	18 (31.6 ± 1.5)[a]
0.036 ppm	60	32 (53.5 ± 3.5)	17 (28.1 ± 3.5)[a]
0.36 ppm	59	32 (54.8 ± 4.5)	17 (28.8 ± 1.1)[a]
3.6 ppm	58	27 (46.2 ± 2.6)	1 (1.7 ± 1.7)[b]
7.2 ppm	53	26 (49.4 ± 3.1)	0 (0)[b]

After 24 h of IVF, the cleaved 2-cell embryos were cultured in SOFaa supplemented with 0.036, 0.36, 3.6, and 7.2 ppm Roundup. The control group contained no Roundup. The development to the compaction or blastocyst stage was evaluated at 84 h or 144 h of in vitro culture. The experiment was repeated five times. The percentage of developmental rate was presented as mean ± SEM

[a, b] Within the same column, values with different superscripts were significantly different, $P < 0.001$

*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

Effects of Roundup on in vitro development of bovine preimplantation embryos

The 2-cell embryos were cultured with Roundup at different concentrations (0.45, 0.9, and 1.8 ppm) to examine the effects of Roundup on bovine preimplantation embryo development. The influence of Roundup on bovine embryo development was shown in Table 3. At the 1.8 ppm Roundup group, only 11.7% of cleaved embryos developed to blastocysts. When Roundup concentration was decreased to 0.9 ppm, the blastocyst rate started to rise (21.3%). And when further reduced to 0.45 ppm, the blastocyst rate was close to the control group (27.8% vs. 30.5%). These results suggest that Roundup is detrimental to bovine preimplantation embryo development at far lower concentrations than the agricultural recommended dose. Meanwhile, the effects of Roundup showed an obvious dose-dependent manner.

Table 3*: Effects of Roundup on *in vitro* development of bovine preimplantation embryos

Roundup	No. of 2-cell embryos	No. (%) of embryos developed to	
		Compaction	Blastocyst
No Roundup	164	96 (58.5 ± 2.6)	50 (30.4 ± 1.7)[a]
0.45 ppm	180	96 (53.2 ± 1.0)	50 (27.7 ± 0.9)[a]
0.9 ppm	178	90 (50.3 ± 2.7)	38 (21.2 ± 1.5)[b]
1.8 ppm	180	90 (50.0 ± 2.1)	21 (11.5 ± 1.6)[c]

After 24 h of IVF, the cleaved 2-cell embryos were cultured in SOFaa supplemented with no Roundup (control), 0.45, 0.9, and 1.8 ppm Roundup. The development to the compaction or blastocyst stage was evaluated at 84 h or 144 h of in vitro culture. The experiment was repeated five times. The percentage of developmental rate was presented as mean ± SEM

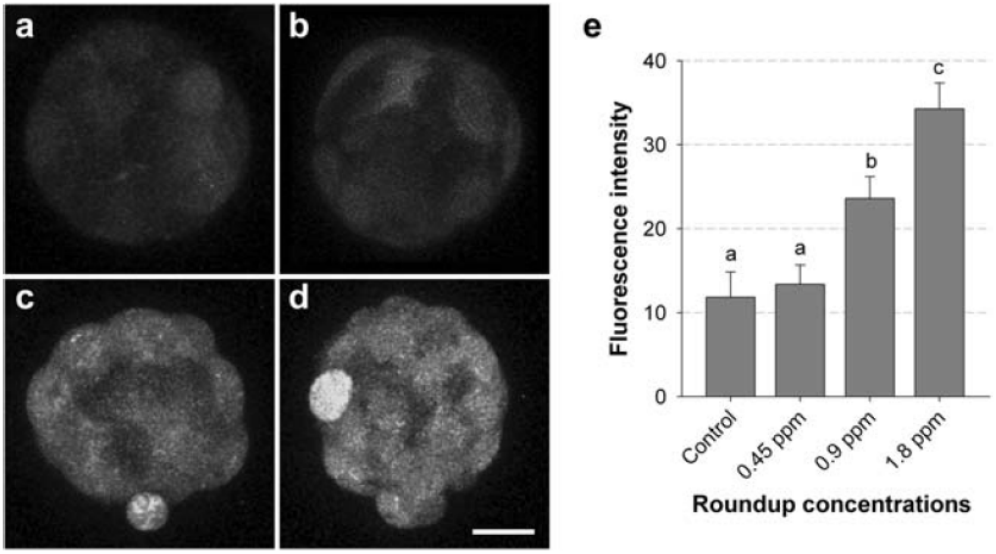
[a, b, c] Within the same column, values with different superscripts were significantly different, $P < 0.05$

*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

Roundup increases intracellular calcium levels in bovine embryos

The 2-cell embryos were cultured with no Roundup (control), 0.45, 0.9, and 1.8 ppm Roundup. After 84 h of *in vitro* culture, the intracellular calcium levels in morulae were detected with Fluo3-AM. Compared with the control group, the embryos from the 0.45 ppm Roundup group showed a moderate increase of intracellular calcium concentration. The embryos cultured with 0.9 ppm Roundup showed significantly higher calcium levels than that in the control and 0.45 ppm Roundup groups. Furthermore, calcium level in the embryos from the 1.8 ppm Roundup group was significantly higher than those from other groups (Fig. 1). These results suggest that Roundup increases the intracellular calcium levels in bovine embryos in a dose-dependent manner.

Fig. 1*: Effects of Roundup on intracellular calcium levels in bovine embryos. The 2-cell embryos were cultured with no Roundup (control), 0.45, 0.9, and 1.8 ppm Roundup. After 84 h of cultivation, the intracellular calcium levels in morulae were measured by Fluo3-AM fluorescence intensity. a)–d) Representative images of intracellular calcium levels in bovine morulae from the control (a), 0.45 (b), 0.9 (c), and 1.8 (d) ppm Roundup groups. Scale bar represented 30 μm. e) The intracellular calcium intensity was measured in the morulae from the control and Roundup groups. From three replicates, 22 morulae were analysed for each group. Each bar denoted mean ± SEM. Superscripts above each bar represented means that differed significantly (P < 0.05)

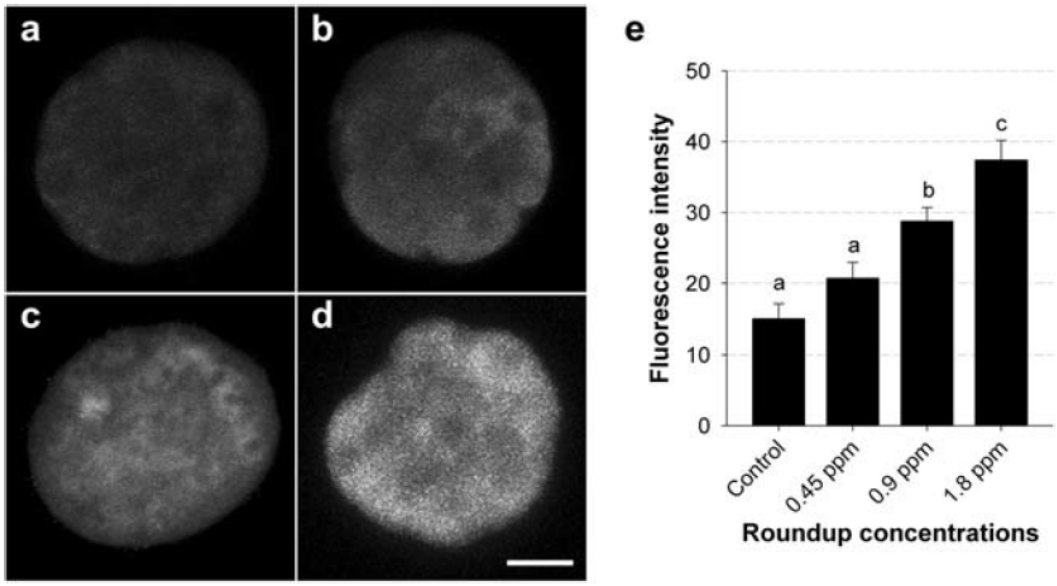


*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

Roundup induces oxidative stress in bovine embryos

The 2-cell embryos were cultured with no Roundup (control), 0.45, 0.9, and 1.8 ppm Roundup. After 84 h of *in vitro* culture, the ROS levels in morulae were measured by DCFH-DA fluorescence intensity. Compared with the control group, the embryos from the 0.45 ppm Roundup group showed a moderate increase of intracellular ROS. The embryos cultured with 0.9 ppm Roundup showed significantly higher levels of ROS than that in control and 0.45 ppm Roundup groups. The ROS level in the embryos from the 1.8 ppm Roundup group was significantly higher than those from other groups (Fig. 2). These results suggest that Roundup induces the intracellular oxidative stress in bovine embryos in a dose-dependent manner.

Fig. 2*: Effects of Roundup on ROS levels in bovine embryos. The 2-cell embryos were cultured with Roundup at different concentrations. After 84 h of cultivation, the ROS levels in morulae were measured by DCFHDA fluorescence intensity. a)–d) Representative images of ROS levels in the morulae from the control (a), 0.45 (b), 0.9 (c), and 1.8 (d) ppm Roundup groups. Scale bar represented 30 μm. e) The fluorescence intensity of ROS was measured in morulae from the control and Roundup groups. From three replicates, 21 morulae were analysed for each group. Each bar denoted mean ± SEM. Superscripts above each bar represented means that differed significantly (P < 0.05)



*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

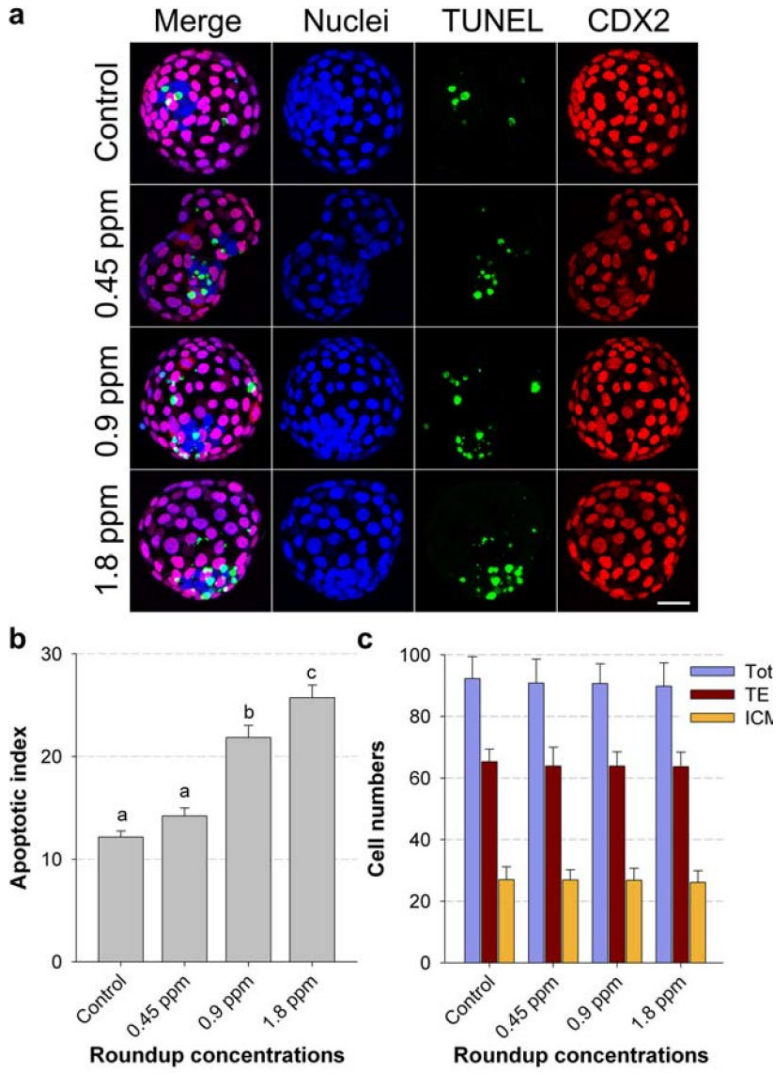
Roundup increases apoptosis in bovine blastocysts

The nuclear DNA fragmentation in blastocysts was detected by TUNEL assay. Increased apoptotic cells were observed in blastocysts treated with Roundup. In the 0.45 ppm Roundup group, only a slight rise of the apoptotic index was observed. Nevertheless, the blastocysts cultured in SOFaa supplemented with 0.9 or 1.8 ppm Roundup had far more apoptotic cells than those in the control and 0.45 ppm Roundup groups. Furthermore, the incidence of apoptosis was positively correlated with Roundup concentrations. More apoptotic cells were observed with the increase of Roundup concentrations (Fig. 3a, b).

Roundup brings no change of cell number in bovine blastocysts

The numbers of ICM and TE cells in bovine blastocysts were counted on by CDX2 immunofluorescence staining. As shown in Fig. 3c, no significant differences were observed in ICM, TE, and total cell numbers among the control and Roundup groups.

Fig. 3*: Effects of Roundup on apoptosis and cell numbers in bovine blastocysts. The 2-cell embryos were cultured with the indicated concentrations of Roundup. Blastocysts were collected at 144 h of cultivation. From five replicates, 21 blastocysts were analysed for each group. The apoptotic nuclei and TE cells were detected by TUNEL assay and CDX2 immunofluorescence, respectively. a) Representative images of blastocysts labelled with TUNEL and CDX2 antibody. The total nuclei were identified by their blue fluorescence. TE cells were identified by the red fluorescence in their nuclei. The fragmented nuclei were recognized by the green fluorescence. Scale bar represents 30 μm. b) The formula for the apoptotic index was (apoptotic cell number/total cell number) × 100. Each bar denoted mean ± SEM. Superscripts above each bar represented means that differed significantly (P < 0.05). c) The ICM, TE, and total cell numbers in the blastocysts from the control and Roundup groups. There were no significant differences in ICM, TE, and total cell numbers among the groups (P > 0.05). Data presented mean ± SD and were analysed by one-way ANOVA



*Retrived from Cai, W., *et al*., Environmental Science and Pollution Research (2020) 27:16451-16459

Conclusion (by the authors)

Our study provide evidence that Roundup in a dose dependent manner is toxic to the quality and *in vitro* development of bovine preimplantation embryos at lower concentrations than agricultural use. Roundup increases the intracellular calcium levels of the embryos and the ROS production is provoked in the embryos exposed to Roundup. Also, Roundup significantly increases the percentage of blastomeres undergoing apoptosis. In summary, Roundup residues from agricultural practices might be more dangerous to mammalian preimplantation embryos than estimated.

Assessment and conclusion

Assessment and conclusion by applicant:
Relevant but supplementary information: The effects of Roundup at 3 doses was investigated on the bovine preimplantation embryo. Direct dosing of formulated product to fertilized embryos *in vitro* is not relevant to real life exposure scenarios. Environmental fate, metabolism and pharmacokinetics for glyphosate active ingredient versus surfactants are very different, and oral co-exposures to mammals at the excessively high doses tested in this case are considered irrelevant to livestock and human health risk assessments. In addition, insufficient information is provided to determine which formulation was tested and whether it is the glyphosate EU representative formulation. No OECD guideline followed, no GLP status stated, no HCD provided and no positive control.

Assessment and conclusion by RMS:
This study shows that Roundup impairs the development and quality of bovine preimplantation embryos in a dose-dependent manner even at 0.9 ppm concentration and that Roundup increases intracellular calcium levels and induces oxidative stress and apoptosis in bovine embryos.

The study is considered as supplementary data. The study is reliable with restrictions because of the following reasons: the test substance is a formulation and insufficient information is provided to determine whether it is the EU representative formulation. No OECD guideline followed, no GLP status and no positive control.

Category B – Abdel-Halim K.Y. *et al.*

Data point	CA 5.9
Report author	Abdel-Halim K. Y. *et al*.
Report year	2019
Report title	Glyphosate and pendimethalin in breast milk samples from Egyptian rural areas: a pilot study for infant's risk assessment
Document source	International Journal of Advanced Research, (2019) Vol. 7, No. 9, pp. 991-1002
Guidelines followed in study	None
Deviations from current test guideline	Not applicable
Previous evaluation	No
GLP/Officially recognised testing facilities	No
Acceptability/Reliability	Not reliable

Aim of the study
The aim of the study was to investigate thirty-one samples of breast milk from rural mothers in Egypt and conducted for the herbicides, glyphosate and pendimethalin analysis followed by their impact on infants.

Materials and methods

Test material:	High purity standards of glyphosate were obtained from Sigma Aldrich Chemie GmbH (Steinheim, Germany) and standard of glyphosate solution were prepared in acetonitrile for chromatography and mass spectrometric analysis.
Lot/Batch:	Not none
Purity:	Not none
Materials:	Acetone, n-hexane, acetonitrile, toluene, anhydrous sodium sulphate and Celite® filter aide, with analytical grade were obtained from BDH Chemical Ltd Co. Trifluoroacetic anhydride (TFAA) and 1-methylimidazole were obtained for Aglient technology, Japan.

Sampling collection and analysis- Study regions
A total of thirty-one (31) nursing mothers were randomly selected for the study from three rural locations, El-Behira governorate; Shobrakhite, Abo Homos and Abo El-Matamir. Another location in Alexandria was selected as a reference zone. The participants were conducted to fill a questionnaire based on personal information and diet status as well as their infant's information. The human breast milk samples were collected in sterilized Falcone tube (45 ml), transferred to the laboratory in ice box and stored at -20°C prior to analyses.

Analytical procedures
Five ml of breast milk were mixed with deactivated Celite® (600°C for 12 hr) in 100 ml centrifuge tube. Ten ml of each solvent system: I (n-hexane: acetone 1:1 v/v); II (n-hexane) and III (n-hexane: acetonitrile 1:1 v/v), respectively, were added. The resulting mixture was agitated by sonication during 20 min followed by centrifugation (2000 rpm; 5 min) for phase's separation. The extraction procedure was repeated. All supernatants were combined, evaporated to dryness and dissolved in 1 ml toluene.

Fluorescent derivatisation of glyphosate was performed in half ml of each sample was mixed with 300 µl of trifluoroacetic anhydride+200 µl of 1-methylimidazole in reaction vail which was sealed with a stopper and mixed well on vortex mixer. The mixture was incubated at 60°C for 1 hr.

Analytes were identified and quantified using High Performance Liquid equipped with an analytical column C18 at room temperature. The mobile phase was used as methanol: acetonitrile 1:1 v/v at a flow rate 1 ml/min. Pendimethalin was subjected to diode array detector at 265 nm, but glyphosate was subjected on fluorescence detector with excitation wavelength 532 nm and emission wavelength 488 nm.

Analytical method validation
The efficiency of the method was conducted via recovery experiment and precision (Coefficient of variation of the results obtained in duplication). The limits of detection (LODs) of the method were calculated. Acceptable recovery percentages were 90.2 and 73.16% for glyphosate with an associated repeatability [Coefficient of variation (CV)] ≤ 20%. Analytes were identified by comparison with retention times of the standard and were quantified with standard solutions.

GC-Mass spectrometry
To confirm the results obtained from HPLC measurement, GC-MS was used. Samples of glyphosate were employed to derivatization as described above. The instrument used was GC-MS equipped with HP 5MS. Helium was used at a flow rate of 2 ml/min. The injection temperature was 160°C. The initial oven temperature was 160°C and increased to 320°C at 6°C/min and held for 2 min. The mass selective detector was operated in EI (electron impact ionization) mode and SIM (selective ion monitoring) mode.

Tolerable daily intake (TDI)
Daily intake of measured herbicides by infants was calculated based on the assumption that the average milk consumption of a 5 kg infant was 250 ml/day (estimated). TDI of herbicides was estimated as follows:'

Where TDI was estimated as mg/kg bw/day, C is the concentration of herbicide (mg/ml milk). Rt is the consumption rate, Cf is the lipid content % (estimated) and Bw is the average body weight of infants (kg).

Statistical analyses
Statistical analyses were performed using COSTAT, Costat User Manual, Version 3. Tucson, Arizona, USA (Cohort Software Inc., 1985). Data were conducted using IBM SPSS statistics 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Herbicide concentrations
The concentrations of herbicides; pendimethalin and glyphosate were measured in breast milk samples collected from three rural locations of El-Behira governorate (Table 1). Samples of Abo El-Matamir location exhibited a range for glyphosate BDL-10.11 µg/ml with mean concentration; 5.24±0.37 µg/ml. The samples collected from Shobrakhite location exhibited a range for glyphosate herbicide was 3.25-27.91 µg/ml attributing mean concentration; 12.66±1.19 µg/ml. Glyphosate concentrations in samples of Abo Homos location ranged from BDL-8.19 µg/ml attributing mean concentration; 6.38±0.28 µg/ml. Alexandria location conducted samples with concentrations lower than those of rural locations arising ranges (3.04-6.98 µg/ml) for glyphosate.

Table 1*: Herbicide concentrations (µg/ml) in breast milk samples of some Egyptian rural residents.

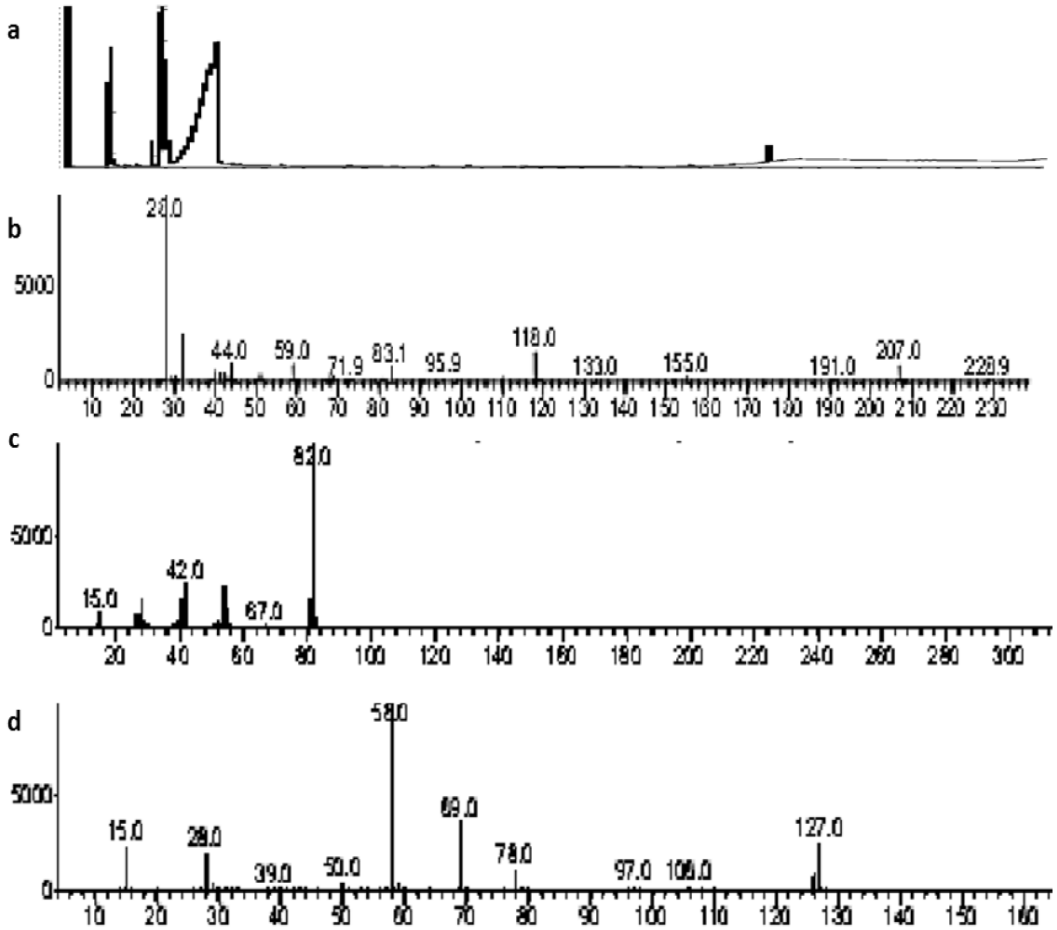
location	(n)	Pendimethalin		Glyphosate	
		range	mean±SD	range	mean±SD
Shobrakhite	8	(1.44-21.63)	5.56±0.86	(3.25-27.91)	12.66±1.19
Abo-Homos	8	(2.51-15.94)	7.80±0.49	(BDL-8.19)	6.38±0.28
Abo El-Matamir	9	(1.88-24.02)	8.05±0.80	(BDL-10.11)	5.24±0.37
Alexandria	6	(BDL-5.30)	4.01±0.15	(3.04-6.98)	4.99±0.27
G. mean	-	-	6.35±1.92	-	7.32±3.61
LSD 0.05	-	-	3.61	-	1.92

BDL=below detection limit; n=number of samples for each location.

*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

Derivatized products of glyphosate were observed under GC separation and detection on mass spectrometer at retention times; 3.00, 3.64, and 5.20 min, respectively, (Fig. 1a), followed by their fragmentation pattern (Fig. 1b). Fluoracylimidzoles is the mode which conducted for glyphosate confirmation on GC-MS. Derivatization reaction for amine compounds give the main products (imidazole and/or N-methyl trifluoroacetamide) (Fig. 1c and d) which are thermal stable.

Fig. 1*: Illustrating (a) GC-MS chromatogram of glyphosate-derivatized products, (b) spectrum pattern of glyphosate-derivatized products, (c) spectrum pattern of the main product 1, imidazole, and (d) the product 2, N-methyl trifluoroacetamide, respectively.



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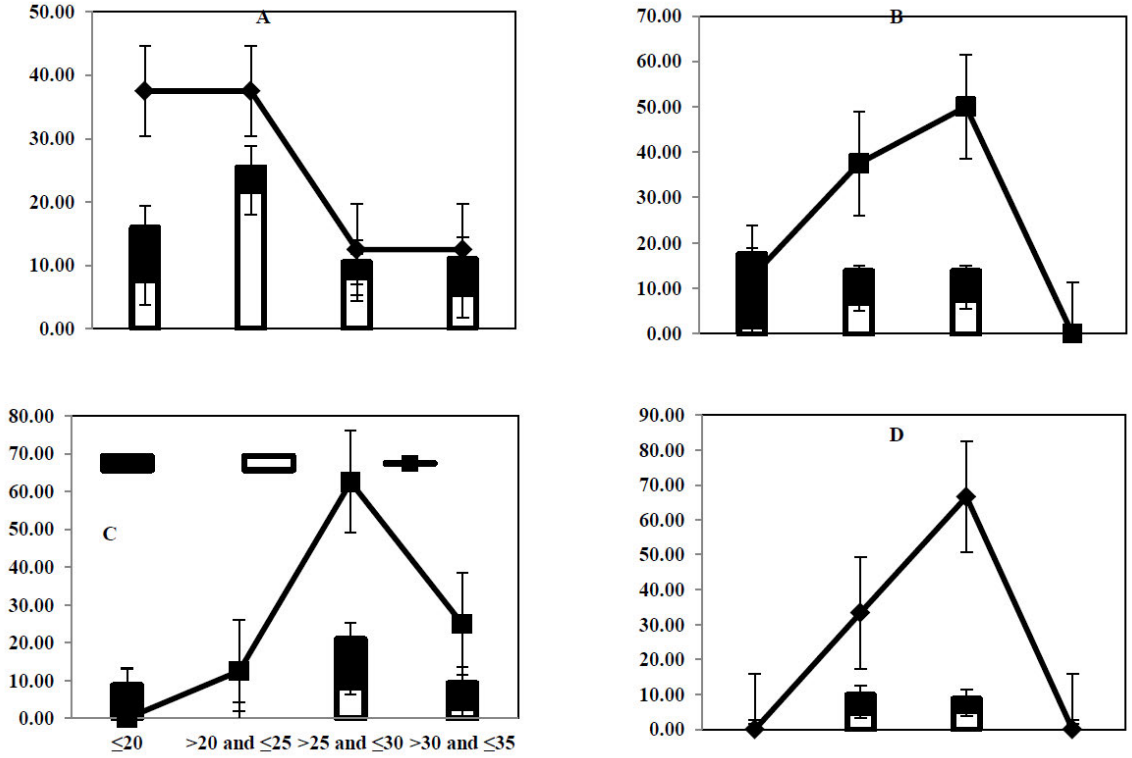
Individual's characteristics in correlation with herbicides

The correlation between the measured herbicides and individual's characteristics were evaluated. The correlation concern herbicide concentrations with lactational mother's age categories are illustrated in Fig. 2. Groups aged≤ 20 yr and (20-25 yr) accounted for 37.5% in Shobrakhite location with mean concentrations; 7.52, 8.46 µg/ml and 21.73, 3.69 µg/ml for glyphosate and pendimethalin, respectively. Other age categories did not exceed

12.5%. Despite group aged≤ 20 yr accounted for 12.5% in Abo Homos, pendimethalin exhibited the greatest mean concentration; 15.94 µg/ml. However, group aged ≤25-≤30 yr accounted for 50.0% arising the greatest concentration of glyphosate; 7.41 µg/ml. Age categories of Abo El-Matamir location exhibited the greatest percentage; 62.5% for the above group with mean concentrations; 8.14 and 12.85 µg/ml for glyphosate and pendimethalin, respectively. In reference location (Alexandria), groups (20-25 yr) and (25-30 yr) accounted for 33.33 and 66.67% with mean concentrations; 4.68, 5.30 and 5.15, 3.58 µg/ml for glyphosate and pendimethalin, respectively. In fact, glyphosate and pendimethalin were negatively associated with mothers age (r=-0.272* and -0.111). The mean ages of locations were in the order as follows: Shobrakhite< Abo Homos< Abo El-Matamir< Alexandria with values: 23.75, 25.00, 29.44 and 27.17 yr, respectively. These age categories exhibited locational mean concentrations; 12.66, 6.38, 5.24, 4.99 µg/ml and 5.56, 7.80, 8.05, 4.01 µg/ml for glyphosate and pendimethalin, respectively.

Fig. 2*: The correlation between herbicide concentrations in human milk and mother age categories in (A) Shborakhit, (B) Abo-Homos, (C) Abo El-Matamir and (D) Alexandria, respectively, (P<0·05)



*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

Consumption frequency for the main diets of studied individuals indicated that, fruits exhibited the greatest percentage 87.5% in moderate level for Abo Homos residents followed by residents of Shobrakhite (75.0%), but individuals of Abo El-Matamir recorded percentage; 75.0% at a high level of consumption. Vegetables exhibited the greatest percentage at high level for individuals of Shobrakhite and Abo Homos locations (100.0 and 87.5%). Moderate level of consumption exhibited the greatest percentages; 75.0 and 83.33% for individuals of Abo El-Matamir and Alexandria locations. Significant different was observed for cereal's consumption. In low level, the greatest percentage; 83.33% was recorded in Alexandria. In moderate level, the greatest value, 75.0% was recorded in Abo El-Matamir location. In high level, the greatest percentages; 100.00 and 87.5% were recorded in Shobrakhite and Abo Homos, respectively. Milk and its products exhibited the greatest percentage; 100.0 and 87.5% (Moderate consumption) for individuals of Shobrakhite and Abo Homos locations. In high level, the greatest percentages 75.0 and 83.33% were recorded for individuals of Abo El-Matamir and Alexandria locations. Meat and fish consumption in rural locations exhibited low and moderate levels compared with Alexandria location (Table 2).

Table 2*: Dietary status and personal information of studied mothers

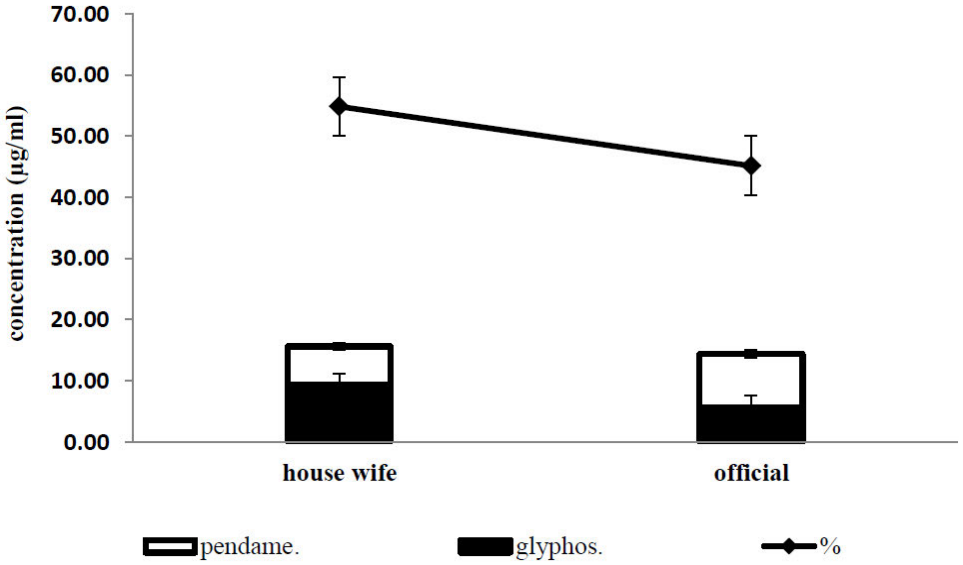
Items	consume	% of dietary intake			
		Shobrakhit	Abo-Homos	Abo El-Matamir	Alexandria
Fruit	Low	0.00	0.00	0.00	16.67
	Moderate	75.00	87.50	25.00	33.33
	high	25.00	12.50	75.00	66.67
Vegetable	Low	0.00	0.00	0.00	16.67
	Moderate	0.00	12.50	75.00	83.33
	high	100.00	87.50	25.00	0.00
Cereal and products	Low	0.00	0.00	0.00	83.33
	Moderate	0.00	12.50	75.00	16.67
	high	100.00	87.50	25.00	0.00
Milk+ its Products	Low	0.00	0.00	0.00	0.00
	Moderate	100.00	87.50	25.00	16.67
	high	0.00	12.50	75.00	83.33
Meats	Low	100.00	87.50	25.00	16.67
	Moderate	0.00	12.50	75.00	33.33
	high	0.00	0.00	0.00	50.00
Fish	Low	100.00	87.50	25.00	0.00
	Moderate	0.00	12.50	75.00	50.00
	high	0.00	0.00	0.00	50.00
Sampling ratio	-	26.67	26.67	26.67	20.00
Average age (yr)	-	23.75	25.00	29.44	27.17
Average No. of infant	-	1.63	1.75	2.00	1.67

*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

Regarding the correlations between herbicide concentrations and individual's intake, glyphosate was positively associated with vegetables (r=0.273*), cereals (r=0.294*), and meats (r=0.055) and negatively with fruits (r=0.020), milk and its products (r=-0.266*), and fish (r=-0.304*), respectively. Pendimethalin was positively associated with fruits (r=0.084), milk and its products (r=0.243*), meat and fish (r=0.055), respectively, and negatively associated with vegetables (r=-0.215*) and cereals (r=-0.055).

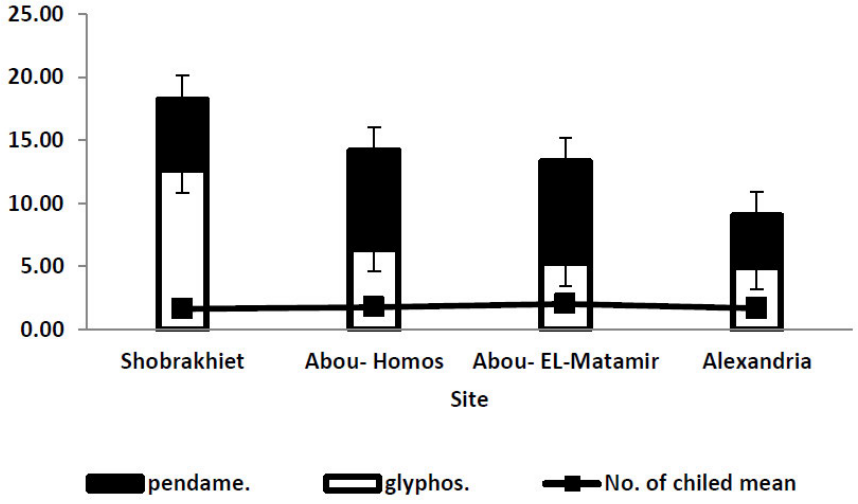
Regarding mother's job, the individuals subjected to 54.84 and 45.16% for house wife and official. Most of house wife individuals live in rural locations, where their samples exhibited mean concentrations of 9.32 and 6.28 µg/ml for glyphosate and pendimethalin, respectively. However, official individuals exhibited mean concentrations of 5.66 and 8.71 µg/ml (Fig. 3). Glyphosate was negatively associated with mother's job (r=-0.330*), but pendimethalin was positively associated with it (r=0.153). The correlation between herbicide concentrations and No. of child or infants was negatively associated with glyphosate concentration (r=-0.216*) and positively associated with pendimethalin (r=0.078) (Fig. 4).

Fig. 3*: The correlation between concentrations of herbicide and mother job



*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

Fig. 4: The correlation between concentrations of herbicides in milk samples and No. of infants



*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

TDIs

Estimated average daily intakes of infants for pendimethalin and glyphosate were addressed for rank of distribution at low, moderate and high levels (Table 3). Infants of Abo El-Matamir location exhibited the greatest value of TDI; 0.19, 2.02, and 8.41 mg/kg/day for pendimethalin exposure, but group of Shobrakhite location exhibited the greatest value; 0.33, 3.17 and 9.77 mg/kg/day for glyphosate. Reference location (Alexandria) exhibited the least values for both herbicides. The rank of exposure for pendimethalin at low level did not exceed 0.25 mg/kg/day at Abo Homos location, but at moderate level it ranged from 1.00 to 2.02 mg/kg/day. At high level, the values were 5.58, 7.57, and 8.41 mg/kg/day at Abo Homos, Shobrakhite, and Abo El-Matamir location, respectively. Regarding glyphosate, rank of distribution reached values; 9.77, 2.87, and 3.54 mg/kg/day for Shobrkhite, Abo Homos, and Abo El-Matamir location, compared with reference location (2.44 mg/kg/day).

Table 3*: Estimated daily intake (DI) of pendimethalin and glyphosate by rural infants

Location	DI (mg/kg/day)					
	pendimethalin			glyphosate		
	low	moderate	high	low	moderate	high
Shobrakhite	0.14	1.39	7.57	0.33	3.17	9.77
Abo Homos	0.25	1.95	5.58	Missing data	1.60	2.87
Abo El-Matamir	0.19	2.02	8.41	Missing data	1.31	3.54
Alexandria	Missing data	1.00	1.86	0.30	1.25	2.44

*Retrived from Abdel-Halim, K. Y., *et al*., Int. J. Adv. Res. 7(9), 991-1002 (2019)

Conclusion (by the author)

The present finding demonstrated a concept about impact of glyphosate and pendimethalin on rural mothers and their infants in Egypt. Positively detection of these compounds in collected breast milk samples was conducted depending on appropriate method followed by good precision and validated chromatographic and mass spectrometric techniques. The young and housewife mothers were the most impacted with herbicides, leading to extensive lactational transfer of them. Thus, TDI of these compounds for infants of the studied participants indicate signs alarming of adverse health effects may be imposed especially that concern endocrine disorders, genotoxicity and immune toxic responses.

Assessment and conclusion

Assessment and conclusion by applicant:

Relevant but supplementary information: This article claims that glyphosate was detected in breast milk. There are several technical issues with this study: 1st: The solubility of glyphosate in toluene is reported as only 36 PPM. The highest sample values the paper claims is just under 30 PPM. So if we had roughly 30 PPM of glyphosate in milk and took 5 mL for analysis then the toluene would have to be capable of solubilizing 150 PPM of glyphosate! 2nd: And this is a key issue – the HPLC method lists an excitation wavelength that is higher than the emission wavelength!

There are several studies evaluating whether glyphosate is detectable in cows milk. A study in human breast milk also was conducted and concluded that glyphosate was not detectable.

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11. Zhao, J., Pacenka, S., Wu, J., Richards, B. K., Steenhuis, T., Simpson, K., & Hay, A. G. (2018). Detection of glyphosate residues in companion animal feeds. Environmental Pollution, 243(Pt B), 1113-1118. https://doi.org/10.1016/j.envpol.2018.08.100

The article is not reliable.

Assessment and conclusion by RMS:

The RMS consider this study non-reliable.
Due to the below reasons:

1 The solubility of glyphosate in toluene is 36 mg/L. The highest value in this paper is just below 30 ppm. If correct, 5 ml of breast milk, extracted, evaporated and dissolved in 1 ml of toluene, the solubility of glyphosate must be approximately 150 ppm and 5 times higher than the reported solubility for glyphosate in toluene.

2. The HPLC method lists an excitation wavelength that is higher than the emission wavelength. According to Stokes law, the emitted light is always of longer wavelength than the excitation light.

Appendix 1: Overview of publications related to reproductive toxicity that are classified by the applicant as "non-relevant after detailed assessment of full-text article"

To complement the standard reproductive toxicity studies, the applicant has performed a literature search in accordance with the EFSA Guidance document EFSA Journal 2011;9(2):2092 “Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) 1107/2009”. The results were categorized as “non-relevant”, as “potentially relevant” or to be of “unclear relevance” following a rapid assessment. For the two latter categories, the full-text documents were reviewed in detail and then categorized as “non-relevant” or “relevant”. The articles considered relevant were categorized as A (providing data for establishing or refining risk assessment parameters), as B (articles relevant to the data requirement but in opinion of the applicant only to provide supplementary information that does not alter existing risk assessment) or as C (articles of unclear relevance).

Reproductive toxicity

Upon review of the titles and abstracts of articles assigned this category, study summaries were requested for the studies listed in the table below to further justify the categorisation of the information. The justification provided by the applicant was reviewed by the RMS and the overall conclusions are presented in the table below.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
1251	Abarikwu S. O. *et al.*	2015	Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats.	Toxicology mechanisms and methods (2015), Vol. 25, No. 1, pp. 70-80	No OECD guideline and GLP status. Both formulations and active substance were used. No source, purity or batch/lot described for active substance. Only one dose tested, and few animals used. Administration only 3 times per week.	*"Background* The potential toxicity resulting from the possible interactions of the herbicides, Ultrazin (atrazine, ATZ) and Bretmont Wipeout (glyphosate, GLY) (as commercialized in Nigeria), is not completely known. We therefore evaluated reproductive- and hepato-toxicity in rats co-exposed to ATZ and GLY. *Methods* Six weeks old male rats were exposed by gavage three times per week to ATZ (12.5 mg/kg) or GLY (5 mg/kg) alone or in combination (12.5 mg/kg ATZ + 5 mg/kg GLY) or vehicle (corn oil), for 52 days. *Results* ATZ and GLY impaired sperm quality but GLY has more adverse effect on sperm quality than ATZ. Testosterone level, sperm motility, sperm counts, live/dead ratio and the weight of the epididymis were lower in the GLY group compared to the ATZ group by 57%, 33%, 20%, 22% and 41% and higher by 109%, 76.7%, 39.6%, 32.3% and 100% respectively in the combine-exposure group (ATZ + GLY) compared to the GLY group. Oxidative stress and histopathological changes were also noticeable in the liver but not in the testis of GLY-treated animals, and the observed effects were more remarkable in the GLY group than the ATZ or the combined-exposure group. The combined effects of the active ingredients on testosterone level, sperm count and hepatic malondialdehyde (MDA) levels were also similar as when the commercial formulations were used. *Conclusion* There are therefore antagonistic interactions between the two toxicants on the toxicity endpoints investigated in this	Not relevant by full text: Formulation provided to Wistar rats via oral gavage in corn oil. The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study is a 450 g/L product differing in components to the EU representative formulation MON 52276. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	The RMS does not agree with the applicant's justification. In this paper also experiments with the active substance were performed. It is known that various adjuvants present in commercial formulations can influence the toxicity, however, they are unlikely to have any influence on the investigated endpoints, as the active substance also produced similar effects as the commercial formulation (as stated by study authors). The RMS considers the study relevant but reliable with restrictions because the glyphosate used is not sufficiently characterised, no analysis of achieved dose level, few animals were used, administration only three times per week, only one dose tested, and no details about clinical signs. <u>Number of animals used in the study:</u> 5 males/group A **data gap** is identified (detailed study summary is missing)

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						study and these effects are due to the active ingredients of both herbicides in the commercial formulations."		
1256	Alarcon R. *et al.*	2019	Neonatal exposure to a glyphosate-based herbicide alters the histofunctional differentiation of the ovaries and uterus in lambs.	Molecular and cellular endocrinology (2019), Vol. 482, pp. 45-56	No OECD guideline and GLP status. A formulation was used, only one dose tested, and few animals were used. No positive control	"The aim of the present study was to compare the effect of oral and subcutaneous exposure to a glyphosate-based herbicide (GBH) on the female reproductive system, specifically in the ovaries and uterus of prepubertal lambs. To this end, ewe lambs were exposed to a s.c. (n: 5) or an oral (n: 5) environmentally relevant dose of GBH (2 mg/kg/day) or to vehicle (controls, n: 12), from postnatal day (PND) 1 to PND14. Serum glyphosate and aminomethylphosphonic acid (AMPA) concentrations were measured on PND15 and PND45. The ovaries and uterus were obtained and weighed on PND45. Ovarian follicular dynamics and uterine morphological features were determined by picrosirius-hematoxylin staining. The proliferation marker Ki67 was evaluated by immunohistochemistry in ovarian and uterine samples. Glyphosate but not AMPA was detected in serum of exposed lambs on PND15, whereas neither glyphosate nor AMPA were detected on PND45. Controls were negative for glyphosate and AMPA on PND15 and PND45. GBH exposure did not affect ovarian or uterine weight. However, on PND45, the ovary of GBH-exposed lambs showed altered follicular dynamics, increased proliferation of granulosa and theca cells, and decreased mRNA expression of FSHR and GDF9, whereas their uterus showed decreased cell proliferation but no alterations in the histomorphology or gene expression. In	Not relevant by full text: Formulation tested (Roundup Full II, Argos SRL, Santa Fe, Argentina; 54 g/100 mL glyphosate) is not the EU representative formulation, therefore the article is not relevant for glyphosate EU renewal. The authors state they could not attribute any observed adverse effects to glyphosate, the co-formulants or the mixture.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. Furthermore, it could be noted that effects on ovaries or uterus were not reported in the standard reproductive toxicity studies conducted with rats.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						conclusion, GBH exposure altered the ovarian follicular dynamics and gene expression, and the proliferative activity of the ovaries and uterus of lambs. It is noteworthy that all the adverse effects found in the ovaries and uterus of both GBH exposed groups were similar, independently of the administration route."		
1257	Altamirano G. A. *et al.*	2018	Postnatal exposure to a glyphosate-based herbicide modifies mammary gland growth and development in Wistar male rats.	Food and chemical toxicology (2018), Vol. 118, pp. 111-118	No OECD guideline and GLP status. A formulation was used and only one dose tested. No positive control.	"Our aim was to evaluate whether postnatal exposure to a glyphosate-based herbicide (GBH) modifies mammary gland development in pre- and post-pubertal male rats. From postnatal day 1 (PND1) to PND7, male rats were injected subcutaneously every 48 h with either saline solution (vehicle) or 2 mg GBH/kg·bw. On PND21 and PND60, mammary gland and blood samples were collected. Estradiol (E2) and testosterone (T) serum levels, mammary gland histology, collagen fiber organization, mast cell infiltration, proliferation index, and estrogen (ESR1) and androgen receptor (AR) expression levels were evaluated. At PND21, GBH-exposed male rats exhibited greater development of the mammary gland with increased stromal collagen organization and terminal end buds (TEBs) compared to control rats. At PND60, the number of TEBs remained high and was accompanied by an increase in mast cell infiltration, proliferation and ESR1 expression in GBH-exposed male rats. In contrast, no effects were observed in E2 and T serum levels and AR expression in both days studied. Our results showed that a postnatal subacute treatment with GBH induces endocrine-disrupting	Not relevant by full text: Formulation tested (Roundup FULL II, potassium salt; 54% a.e.). The formulation tested contains potassium salts and co formulants not directly comparable to the EU representative formulation MON 52276, therefore the article is not relevant for the glyphosate EU renewal. The authors state that additional studies are needed to demonstrate whether effects on male mammary glands are a potential result of glyphosate alone or in combination with adjuvants. The subcutaneous dosing to developing rats does not directly simulate anticipated operator or consumer exposure routes. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. Furthermore, it could be noted that effects on mammary gland were not reported in the standard reproductive toxicity studies. Number of animals used in the study: 8-10 male pups/per group.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						effects in the male mammary gland *in vivo*, altering its normal development."		
1260	Anifandis G. *et al.*	2017	The *In vitro* Impact of the Herbicide Roundup on Human Sperm Motility and Sperm Mitochondria.	Toxics (2017), Vol. 6, No. 1, pp. 2	No OECD guideline and GLP status. A formulation was used and only one dose tested. Direct exposure and no positive control. Test substance not sufficiently characterized.	"Toxicants, such as herbicides, have been hypothesized to affect sperm parameters. The most common method of exposure to herbicides is through spraying or diet. The aim of the present study was to investigate the effect of direct exposure of sperm to 1 mg/L of the herbicide Roundup on sperm motility and mitochondrial integrity. Sperm samples from 66 healthy men who were seeking semen analysis were investigated after written informed consent was taken. Semen analysis was performed according to the World Health Organization guidelines Mitochondrial integrity was assessed through mitochondrial staining using a mitochondria-specific dye, which is exclusively incorporated into functionally active mitochondria. A quantity of 1 mg/L of Roundup was found to exert a deleterious effect on sperm's progressive motility, after 1 h of incubation (mean difference between treated and control samples = 11.2%) in comparison with the effect after three hours of incubation (mean difference = 6.33%, $p < 0.05$), while the relative incorporation of the mitochondrial dye in mitochondria of the mid-piece region of Roundup-treated spermatozoa was significantly reduced compared to relative controls at the first hour of incubation, indicating mitochondrial dysfunction by Roundup. Our results indicate that the direct exposure of semen samples to the active constituent of the herbicide Roundup at the relatively low concentration of 1 mg/L has adverse effects on sperm motility, and this may	Not relevant by full text: Formulation tested *in vitro* (Roundup, not characterized). Participants in the study were apparently not screened for any previous exposures or environmental factors prior to the study and the specific motivations for their involvement in the study was not reported. No apparent evaluation of the quality or motility of the sperm was assessed prior to the study and this evaluation is important to determine if the participants had underlying health conditions that affected semen quality or why they were seeking semen analysis potentially due to infertility or other pre-existing health conditions. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. In addition, the test substance was not characterized. Also, the relevance of direct exposure for the *in vivo* situation cannot be established. The study is not considered further for the endpoint of reproductive toxicity.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						be related to the observed reduction in mitochondrial staining."	secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.	
1269	Avdatek F. *et al.*	2018	Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats.	Andrologia (2018), Vol. 50, No. 7, pp. e13036	No OECD guideline and GLP status. A formulation was used, only one dose tested and few animals used.	"The aim of this study was to examine the protective effect of N-acetylcysteine (NAC) on testicular oxidative damage, spermatological parameters and DNA damage caused by Glyphosate (GLF) in rats. In total, twenty-eight Wistar male rats were evaluated by being separated into four groups in an equal way. Rats in group I, which represented the control group, were fed normal diet without GLF or NAC, group II received normal feed containing 160 mg/kg/daily NAC, group III received normal feed containing 375 mg/kg/daily GLF, and group IV received normal feed containing 160 mg/kg/daily NAC + 375 mg/kg/daily GLF. GLF administration decreased sperm motility, abnormal sperm rate, sperm plasma membrane integrity, glutathione level and superoxide dismutase in the rats' testicular tissue. On the other hand, high malondialdehyde level and DNA damage were detected in the group administered with GLF. Besides, in histopathological terms, a decrease in sperm concentration and degeneration of sertoli cells were determined in the testicular tissue. NAC and NAC+GLF administration reversed lipid peroxidation and DNA damage induced by GLF, the activity of antioxidant enzymes and cell integrity in rats' testis. The above-mentioned findings indicate	Not relevant by full text: Glyphosate based formulation tested (Knockdown 48 SL) contains a different overall composition, which is not comparable to the representative EU formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that no effects on motility were observed for sperms in standard toxicity studies conducted with rats. <u>Number of animals used in the study:</u> 28 Male Wistar rats divided into 4 groups (n=7) (group 1, control group normal diet, group 2, normal diet containing 20 mg/kg bw/day RES, group 3, containing 375 mg/kg bw/day GLY and group 4, 20 mg/kg bw/day RES + 375 mg/kg bw/day GLY)

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						that NAC reduces lipid peroxidation caused by GLF, improves the antioxidant defense mechanism and regenerates tissue damage in rats' testis."		
1270	Avdatek F. *et al.*	2018	Protective effect of N-acetylcysteine on testicular oxidative damage, spermatological parameters and DNA damage in glyphosate-based herbicide-exposed rats.	Kocatepe Veterinary Journal (2018), Vol. 11, No. 3, pp. 292-300	No OECD guideline and GLP status. A formulation was used, only one dose tested, and few animals used.	"The aim of this study was to examine the protective effect of N-acetylcysteine (NAC) on testicular oxidative damage, spermatological parameters and DNA damage caused by Glyphosate (GLF) in rats. In total, twenty-eight Wistar male rats were evaluated by being separated into four groups in an equal way. Rats in group I, which represented the control group, were fed normal diet without GLF or NAC, group II received normal feed containing 160 mg/kg/daily NAC, group III received normal feed containing 375 mg/kg/daily GLF, and group IV received normal feed containing 160 mg/kg/daily NAC + 375 mg/kg/daily GLF. GLF administration decreased sperm motility, abnormal sperm rate, sperm plasma membrane integrity, glutathione level and superoxide dismutase in the rats' testicular tissue. On the other hand, high malondialdehyde level and DNA damage were detected in the group administered with GLF. Besides, in histopathological terms, a decrease in sperm concentration and degeneration of sertoli cells were determined in the testicular tissue. NAC and NAC+GLF administration reversed lipid peroxidation and DNA damage induced by GLF, the activity of antioxidant enzymes and cell integrity in rats' testis. The above-mentioned findings indicate that NAC reduces lipid peroxidation caused by GLF, improves the antioxidant defense mechanism and regenerates tissue damage in rats' testis."	Not relevant by full text: Glyphosate based formulation tested (Knockdown 48 SL) contains a different overall composition, which is not comparable to the representative EU formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that no effects on motility or morphological changes were observed for sperms in standard toxicity studies conducted with rats. <u>Number of animals used in the study:</u> 28 Male Wistar rats divided into 4 groups (n=7) (group 1, control group normal diet, group 2, normal diet containing 160 mg/kg bw/day NAC, group 3, containing 375 mg/kg bw/day GLY and group 4, 160 mg/kg bw/day NAC + 375 mg/kg bw/day GLY)

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
1238	Bhardwaj J. K. *et al.*	2019	Effective attenuation of glyphosate-induced oxidative stress and granulosa cell apoptosis by vitamins C and E in caprines.	Molecular reproduction and development (2019), Vol. 86, No. 1, pp. 42-52	No OECD guideline and GLP status. A formulation was used.	"Pesticides are known to cause a wide range of reproductive problems that possess degenerative effects on mammalian fertility. Glyphosate (GLP), a broad-spectrum organophosphate herbicide, is known to be a potent mammalian toxicant. The present study aims at assessing the GLP-induced (0.1, 2.0, and 4.0 mg/ml) granulosa cells toxicity and evaluating the mitigating effects of vitamins C and E (0.5mM and 1.0 mM) in healthy caprine antral follicles, cultured *in vitro* in a dose and time-dependent manner (24, 48, and 72 hr) and subjected to various cytotoxic and genotoxic analysis, namely, classic histology, EB/AO differential staining, oxidative stress parameters, and antioxidant enzymatic activity. The histomorphological analysis and EB/AO staining elucidated increase in the incidence of apoptotic attributes within granulosa cells with increasing dose and duration of the GLP treatment. The highest apoptotic frequency was observed at 4.0 mg/ml GLP after 72-hr exposure duration in comparison with the control. GLP exposure also led to a significant decline in the antioxidant enzymes' activity, namely, SOD, catalase, and GST along with enhanced lipid peroxidation and reduced FRAP activity in a dose- and	Not relevant by full text: Glyphosate based herbicide tested with *in vitro* test system. The 41% Indian Roundup formulation testing is not comparable in composition and co formulants to the EU representative formulation MON 52276. Therefore, the article is not considered relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. Also, the relevance of direct exposure for the *in vivo* situation cannot be established. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that effects on uterus were not reported in standard reproductive toxicity studies conducted with rats.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						time-dependent manner. Vitamins C and E supplementation decreased oxidative stress-mediated granulosa cells apoptosis, suggesting its efficiency to diminish GLP-mediated GCs cytotoxicity and thereby, preventing associated fertility disorders."		
1298	Clair E. *et al.*	2012	A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels.	Toxicology *in vitro* (2012), Vol. 26, No. 2, pp. 269-79	No OECD guideline and GLP status. Both a glyphosate formulation and glyphosate as active ingredients were used. No purity or batch/lot described. No positive control.	"The major herbicide used worldwide, Roundup, is a glyphosate-based pesticide with adjuvants. Glyphosate, its active ingredient in plants and its main metabolite (AMPA) are among the first contaminants of surface waters. Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Here we tested glyphosate and its formulation on mature rat fresh testicular cells from 1 to 10000 ppm, thus from the range in some human urine and in environment to agricultural levels. We show that from 1 to 48 h of Roundup exposure Leydig cells are damaged. Within 24–48 h this formulation is also toxic on the other cells, mainly by necrosis, by contrast to glyphosate alone which is essentially toxic on Sertoli cells. Later, it also induces apoptosis at higher doses in germ cells and in Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption is a testosterone decrease by 35%. The pesticide has thus an endocrine impact at very low environmental doses, but only a high contamination appears to provoke an acute rat testicular toxicity. This does not anticipate the chronic toxicity which is insufficiently tested, and only with glyphosate in regulatory tests."	Not relevant by full text: This publication is considered not relevant for the glyphosate EU renewal and risk assessment because a glyphosate formulation (Roundup Bioforce) was used for *in vitro* testing instead of glyphosate. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.	In this paper also experiments with the active substance were performed. However, the relevance of direct exposure for the *in vivo* situation cannot be established. The study is not considered further for the endpoint of reproductive toxicity.
1314	de Liz	2013	Roundup disrupts	Free radical	No OECD guideline	"Glyphosate is the primary active	Not relevant by full text:	The RMS does agree with the

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
	Oliveira Cavalli V. L. *et al.*		male reproductive functions by triggering calcium-mediated cell death in rat testis and Sertoli cells.	biology & medicine (2013), Vol. 65, pp. 335-46	and GLP status. Both a glyphosate formulation and glyphosate as active ingredients were used. No purity or batch/lot described.	constituent of the commercial pesticide Roundup. The present results show that acute Roundup exposure at low doses (36 ppm, 0.036 g/L) for 30 min induces oxidative stress and activates multiple stress-response pathways leading to Sertoli cell death in prepubertal rat testis. The pesticide increased intracellular Ca2+ concentration by opening L-type voltage-dependent Ca2+ channels as well as endoplasmic reticulum IP3 and ryanodine receptors, leading to Ca2+ overload within the cells, which set off oxidative stress and necrotic cell death. Similarly, 30 min incubation of testis with glyphosate alone (36 ppm) also increased 45Ca2+ uptake. These events were prevented by the antioxidants Trolox and ascorbic acid. Activated protein kinase C, phosphatidylinositol 3-kinase, and the mitogen-activated protein kinases such as ERK1/2 and p38MAPK play a role in eliciting Ca2+ influx and cell death. Roundup decreased the levels of reduced glutathione (GSH) and increased the amounts of thiobarbituric acid-reactive species (TBARS) and protein carbonyls. Also, exposure to glyphosate– Roundup stimulated the activity of glutathione peroxidase, glutathione reductase, glutathione S-transferase, γ-glutamyltransferase, catalase, superoxide dismutase, and glucose-6-phosphate dehydrogenase, supporting downregulated GSH levels. Glyphosate has been described as an endocrine disruptor affecting the male reproductive system; however, the molecular basis of its toxicity remains to be clarified. We propose that Roundup toxicity, implicated in Ca2+ overload, cell signaling misregulation, stress	Formulation tested *in vitro* was Roundup Original, 360 g/L a.e., Brazil. As this formulation differs in composition and co formulants to the representative formulation MON 52276, the article is not considered relevant to the EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. Also, the relevance of direct exposure for the *in vivo* situation cannot be established. The study is not considered further for the endpoint of reproductive toxicity.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						response of the endoplasmic reticulum, and/or depleted antioxidant defenses, could contribute to Sertoli cell disruption in spermatogenesis that could have an impact on male fertility."		
1337	Emmanuel A. G. *et al.*	2015	Protective potential of betulinic acid against glyphosate-induced toxicity in testis and epididymis of male wistar rats	International Journal of Current Research (2015), Vol. 7, No. 6, pp. 16650-16660	No OECD guideline and GLP status. Commercial glyphosate of unknown source was used, route of administration not stated and dosing 4 times per 14 day at day 4, 6, 10 and 12.	"Betulinic acid (BA), a novel pentacyclic triterpene, widely distributed in plants. This study examined possible protective potentials of Betulinic acid on antioxidant parameters and histology of testis and epididymis of glyphosate treated rats. Glyphosate significantly elevated Malondialdehyde (MDA) in testes and epididymis by 41.8 % and 49 5%, respectively, compared with controls. BA significantly decreased MDA by 47.8% and 34.0%, respectively, compared with glyphosate group. Superoxide dismutase (SOD) was significantly reduced by 66.7% and 73.7% in testes and epididymis in the glyphosate group, whereas BA supplementation significantly elevated ($p < 0.05$) SOD by 77.8% and 72.2%, respectively. Catalase (CAT) activities were reduced in the glyphosate group by 29.2% and 21.7% in testes and epididymis, while BA elevated CAT activities by 23.8% and 28.0%, respectively. Glyphosate reduced, reduced glutathione (GSH) level by 35.2% in testes, compared with controls, while BA supplementation increased GSH level by 43.8%, compared with the glyphosate group. Glyphosate induced cellular degeneration and deformity in testis and epididymis and supplementation with BA was observed to reverse these effects. From the results, glyphosate disturbed the antioxidant defense system and histological features of testicular and epidymal tissues, while	Not relevant by full text: Formulation tested *in vivo* was described as "commercial glyphosate". The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. <u>Number of animals and dose levels used in study:</u> In this study, twenty male Wistar rats (185 ± 2.5g) rats were purchased and acclimatized for 7 days and then randomly divided into groups; four groups (A- D) with five rats in each group. Rats in group A served as the control, the rats in group B were administered with betulinic acid (10 mg/kg) every other day, group C was treated with glyphosate (100 mg/kg) on days 4, 6, 10 and 12, while group D was pretreated with betulinic acid (10 mg/kg) on days 1 and 3, and continued every other day, with glyphosate (100 mg/kg) on days 4, 6, 10 and 12. After 14 days of treatment, rats were fasted overnight and weighed. Blood was collected by ocular bleeding and rats were

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						Betulinic acid exhibited the potential to prevent these toxic effects in male rats."		sacrificed by cervical dislocation.
1365	Guerrero Schimpf M. *et al.*	2017	Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus.	Toxicology (2017), Vol. 376, pp. 2-14	No OECD guideline and GLP status. Commercial glyphosate formulation used, only one dose tested, few animals used, dosing every 48 h and no positive control used.	"Glyphosate-based herbicides (GBHs) are extensively used to control weeds on both cropland and noncropland areas. No reports are available regarding the effects of GBHs exposure on uterine development. We evaluated if neonatal exposure to a GBH affects uterine morphology, proliferation and expression of proteins that regulate uterine organogenetic differentiation in rats. Female Wistar pups received saline solution (control, C) or a commercial formulation of glyphosate (GBH, 2 mg/kg) by sc injection every 48 h from postnatal day (PND) 1 to PND7. Rats were sacrificed on PND8 (neonatal period) and PND21 (prepubertal period) to evaluate acute and short-term effects, respectively. The uterine morphology was evaluated in hematoxylin and eosin stained sections. The epithelial and stromal immunophenotypes were established by assessing the expression of luminal epithelial protein (cytokeratin 8; CK8), basal epithelial proteins (p63 and pan cytokeratin CK1, 5, 10 and 14); and vimentin by immunohistochemistry (IHC). To investigate changes on proteins that regulate uterine organogenetic differentiation we evaluated the expression of estrogen receptor alpha (ERa), progesterone receptor (PR), Hoxa10 and Wnt7a by IHC. The GBH-exposed uteri showed morphological changes, characterized by an increase in the incidence of luminal	Not relevant by full text: Formulation tested *in vivo* via subcutaneous injection (Roundup FULL II, 66.2% potassium salt). As this is not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that effects on uterus were not reported in standard reproductive toxicity studies in the rat. <u>Strain and number of animals used:</u> Female Wistar pups (8 per group)

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						epithelial hyperplasia (LEH) and an increase in the stromal and myometrial thickness. The epithelial cells showed a positive immunostaining for CK8, while the stromal cells for vimentin. GBH treatment increased cell proliferation in the luminal and stromal compartment on PND8, without changes on PND21. GBH treatment also altered the expression of proteins involved in uterine organogenetic differentiation. PR and Hoxa10 were deregulated both immediately and two weeks after the exposure. ERa was induced in the stromal compartment on PND8, and was downregulated in the luminal epithelial cells of glyphosate-exposed animals on PND21. GBH treatment also increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. Neonatal exposure to GBH disrupts the postnatal uterine development at the neonatal and prepubertal period. All these changes may alter the functional differentiation of the uterus, affecting the female fertility and/or promoting the development of neoplasias."		
1394	Ikpeme E. V. *et al.*	2012	Efficacy of ascorbic acid in reducing glyphosate-induced toxicity in rats.	British Biotechnology Journal (2012), Vol. 2, No. 3, pp. 157-168	No OECD guideline and GLP status. Glyphosate used not described and source and brand unknown. Strain unknown, few animals used, and only two doses tested.	"Aim: Humans and animals interact with their environments on a daily basis and, as a consequence, are exposed to a broad spectrum of synthesized chemicals present in the food they eat, the air they breathe and the water they drink including glyphosate. This study was aimed at investigating the effects of glyphosate on the sperm dynamics of male albino rats and the protective effects of ascorbic acid. Methods: Twenty five mature male albino rats were weighed and divided into five groups in a completely randomized design (CRD). Group 1 rats	Not relevant by full text: The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the	The test substance was not sufficiently characterized. It is not clear if the test substance was a formulation or the active substance. Due to the limitations and uncertainties in the study, the relevance of the results is difficult to assess. The study is not considered further for the endpoint of reproductive toxicity.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						served as the control. Rats in groups 2 and 4 received 250 ml/kg and 500 ml/kg of glyphosate while groups 3 and 5 rats were administered with 250 ml/kg and 500 ml/kg of glyphosate and 200 mg/kg of ascorbic acid, respectively, which were administered orally using oral gavages. The treatment regimen lasted for 65 days. Results: Our results showed that there were significant adverse effects ($P<0.05$) of glyphosate treatment on sperm parameters and the cyto-architecture of the gonad, which showed disruption in the seminiferous tubules, necrotic germinal epithelium and clumped Leydig cells. However, administering the rats with ascorbic acid caused significant ameliorating effects on the parameters investigated. Conclusion: Succinctly, glyphosate exposure to animals is detrimental to their reproductive physiology, including the cellular integrity of the gonads. This not notwithstanding, administering the affected animals with ascorbic acids might reduce the toxicity inflicted by the glyphosate."	formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.	
1396	Ingaramo P. I. *et al.*	2019	Acute uterine effects and long-term reproductive alterations in postnatally exposed female rats to a mixture of commercial formulations of endosulfan and glyphosate.	Food and chemical toxicology (2019), Vol. 134, pp. 110832	No OECD guideline and GLP status. Glyphosate formulation source and brand unknown. Only one dose tested and no positive control used.	"Endosulfan and glyphosate are widely used pesticides and have been associated to reproductive disorders. We examine the acute and long-term effects of postnatal exposure to commercial formulations of endosulfan (EF), glyphosate (glyphosate-based herbicide, GBH) and a mixture of both pesticides (MIX). After birth, female pups of Wistar rats received saline solution (CONTROL), EF (600 µg/kg of b.w/day), GBH (2 mg/kg of b.w/day) or a mixture (at the same doses) from postnatal day (PND) 1 to PND7. The uterine histology and expression of	Not relevant by full text: A glyphosate based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that effects on uterus were not reported in standard reproductive toxicity studies.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						Hoxa10, estrogen (ERα) and progesterone (PR) receptors were evaluated on PND8. Reproductive performance was evaluated on gestational day 19. GBH and MIX rats showed an increment of 1) the incidence of luminal epithelial hyperplasia, 2) PR and Hoxa10 expression. EF modified ERα and Hoxa10 expression. During adulthood, MIX and GBH rats showed higher post-implantation losses while EF alone produced an increase of preimplantation losses. We showed that the co-administration of both pesticides produced acute uterine effects and long–term deleterious reproductive effects that were similar to those induced by GBH alone. We consider important to highlight the necessity to evaluate the commercial pesticide mixture as a more representative model of human exposure to a high number of pesticides."	to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.	Number of animals used in the study: Female pups of Wistar rats received by s.c. administration saline solution (control n=34), EF (600 µg/kg of bw/day, n=38), GBH (2 mg/kg of bw/day, n=38) or a mixture at the same doses (n=38) from PND 1 to PND7.
1397	Ingaramo P. I. *et al.*	2017	Neonatal exposure to a glyphosate-based herbicide alters uterine decidualization in rats.	Reproductive toxicology (2017), Vol. 73, pp. 87-95	No OECD guideline and GLP status. Glyphosate formulation source and brand unknown. Only one dose tested and no positive control used.	"We investigated whether defective modulation of uterine signaling may cause decidualization failure in rats neonatally exposed to a glyphosate-based herbicide (GBH). Female pups received vehicle or 2 mg/kg of GBH from postnatal day (PND) 1 to PND7. On PND8 and PND21, Wnt5a and β-catenin expression was evaluated in uterine samples. On gestational day (GD) 9, Wnt5a, Wnt7a and β-catenin expression and Dkk1 and sFRP4 mRNA were evaluated on implantation sites. On PND8, GBH-exposed rats showed increased Wnt5a and β-catenin expression in luminal epithelium (LE), whereas on PND21, they showed increased Wnt5a and β-catenin	Not relevant by full text: A glyphosate based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that effects on uterus were not reported in standard reproductive toxicity studies. Strain and number of animals used in the study: Female pups were assigned to two neonatal treatment

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						expression in subepithelial stroma but decreased β-catenin expression in glandular epithelium. On GD9, GBH-exposed rats showed decreased Wnt5a and Wnt7a expression in the antimesometrial zone and LE respectively, without changes in β-catenin expression, while Dkk1 and sFRP4 were up- and down-regulated respectively. We concluded that neonatal GBH exposure may lead to embryo losses by disturbing uterine signaling."	glyphosate exposure or of one of the other components.	groups: 1) the control group, receiving saline solution (n = 34); and 2) the GBH group, receiving a commercial formula-tion of glyphosate dissolved in saline solution at 2 mg/kg (n = 38).
1398	Ingaramo P. I. *et al.*	2016	Effects of neonatal exposure to a glyphosate-based herbicide on female rat reproduction.	Reproduction (2016), Vol. 152, No. 5, pp. 403-15	No OECD guideline and GLP status. Glyphosate formulation source and brand unknown. Only one dose tested and the dosing only every second day.	"In this study, we investigated whether neonatal exposure to a glyphosate-based herbicide (GBH) alters the reproductive performance and the molecular mechanisms involved in the decidualization process in adult rats. Newborn female rats received vehicle or 2 mg/kg/day of a GBH on postnatal days (PND) 1, 3, 5 and 7. On PND 90, the rats were mated to evaluate (i) the reproductive performance on gestational day (GD) 19 and (ii) the ovarian steroid levels, uterine morphology, endometrial cell proliferation, apoptosis and cell cycle regulators, and endocrine pathways that regulate uterine decidualization (steroid receptors/COUP-TFII/Bmp2/ Hoxa10) at the implantation sites (IS) on GD9. The GBH-exposed group showed a significant increase in the number of resorption sites on GD19, associated with an altered decidualization response. In fact, on GD9, the GBH-treated rats showed morphological changes at the IS, associated with a decreased expression of estrogen and progesterone receptors, a downregulation of COUP-TFII (Nr2f2) and Bmp2 mRNA and an increased	Not relevant by full text: A glyphosate based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.	The RMS does agree with the applicant's justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that effects on uterus were not reported in standard reproductive toxicity studies. <u>Strain and number of animals used in the study:</u> Strain of rats: an inbred Wistar-derived strain Female pups from each foster mother were assigned to one of the following neonatal treatment groups: (1) control group (C), receiving saline solution (n = 34); and (2) GBH group, receiving a commercial formulation of glyphosate dissolved in saline solution at 2 mg/kg (n = 38).

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						expression of HOXA10 and the proliferation marker Ki67(Mki67) at the IS. We concluded that alterations in endometrial decidualization might be the mechanism of GBH-induced post-implantation embryo loss."		
1405	Jiang X. *et al.*	2018	A commercial Roundup formulation induced male germ cell apoptosis by promoting the expression of XAF1 in adult mice	Toxicology Letters (2018), Vol. 296, pp. 163-172	No OECD guideline and GLP status. Glyphosate formulation used, male mice treated for 35 days only.	"Roundup® is extensively used for weed control worldwide. Residues of this compound may lead to side effects of the male reproductive system. However, the toxic effects and mechanisms of Roundup® of male germ cells remain unclear. We aimed to investigate the apoptosis-inducing effects of Roundup® on mouse male germ cells and explore the role of a novel tumor suppressor XAF1 (X-linked inhibitor of apoptosis-associated factor 1) involved in this process. We demonstrated that Roundup® can impair spermatogenesis, decrease sperm motility and concentration, and increase the sperm deformity rate in mice. In addition, excessive apoptosis of germ cells accompanied by the overexpression of XAF1 occurred after Roundup® exposure both *in vitro* and *in vivo*. Furthermore, the low expression of XIAP (X-linked inhibitor of apoptosis) induced by Roundup® was inversely correlated with XAF1. Moreover, the knockdown of XAF1 attenuated germ cell apoptosis, improved XIAP expression and inhibited the activation of its downstream target proteins, caspase-3 and PARP, after Roundup® exposure. Taken together, our data indicated that XAF1 plays an important role in Roundup®-induced male germ cell apoptosis. The present study suggested that Roundup® exposure has potential	Not relevant by full text: In this study, a Roundup formulation containing polyethoxylated tallow amine (POEA) was administered via gavage to adult male mice. The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains polyoxyethylene tallow amine (POEA), which is not permitted for use in formulated herbicidal products in the EU.	The RMS does agree with the applicant's justification. The test substance was a formulation (containing POEA which is not permitted for use in EU) and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could be noted that no morphological changes or effects on motility were observed for sperms in standard toxicity studies conducted with rats. <u>Strain and number of animals used in the study:</u> Adult 8-week-old male Kunming mice approximately 34 g were randomly allocated into 4 groups (n=8 per group), control, 60, 180 and 540 mg/kg glyphosate.

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						negative implications on male reproductive health in mammals."		
1414	Kubsad D. *et al.*	2019	Assessment of Glyphosate Induced Epigenetic Transgenerational Inheritance of Pathologies and Sperm Epimutations: Generational Toxicology	Scientific Reports (2019), Vol. 9, No. 1, pp. 1-17	No OECD guideline and GLP status. Glyphosate purity, lot/batch not described. Dose given intraperitoneally. Only one dose tested.	Ancestral environmental exposures to a variety of factors and toxicants have been shown to promote the epigenetic transgenerational inheritance of adult onset disease. One of the most widely used agricultural pesticides worldwide is the herbicide glyphosate (N-(phosphonomethyl)glycine), commonly known as Roundup. There are an increasing number of conflicting reports regarding the direct exposure toxicity (risk) of glyphosate, but no rigorous investigations on the generational actions. The current study using a transient exposure of gestating F0 generation female rats found negligible impacts of glyphosate on the directly exposed F0 generation, or F1 generation offspring pathology. In contrast, dramatic increases in pathologies in the F2 generation grand-offspring, and F3 transgenerational greatgrand- offspring were observed. The transgenerational pathologies observed include prostate disease, obesity, kidney disease, ovarian disease, and parturition (birth) abnormalities. Epigenetic analysis of the F1, F2 and F3 generation sperm identified differential DNA methylation regions (DMRs). A number of DMR associated genes were identified and previously shown to be involved in pathologies. Therefore, we propose glyphosate can induce the transgenerational inheritance of disease and germline (e.g. sperm) epimutations. Observations suggest the generational toxicology of glyphosate needs to be considered in the disease etiology of future generations.	Not relevant by full text: This publication is considered not relevant because the intraperitoneal route of administration is not appropriate for the human health risk assessment of glyphosate, and is associated with dissimilar toxicokinetics when compared oral dosing. Controls were treated with two different dosing vehicles (PBS and DMSO); further, the data provided clearly show that these two vehicles induced different epigenetic alterations in the 1st and 2nd generations of offspring and, therefore, cannot be considered equivalent. Some controls were dropped from the study due to a "founder effect" related to the induction of obesity, one of the primary endpoints evaluated in this study. This deselection of control animals severely confounds and limits interpretation of the study results. Some animals were excluded from individual assessments (e.g., the age at which puberty was attained); however, the reason/basis for their exclusion was not provided and cannot be determined based on the information provided. None of the DNA methylation patterns or phenotypic changes were shown to be	The fact that the dose was given intraperitoneally is not in itself a reason for non-relevance. However, the test substance was not sufficiently characterized. In addition, only one dose was tested. Taking into account that the "pathologies" referred to in F2 and F3 were not seen in the F2 generations of the standard OECD studies in section B 6.8.1, the relevance of this information is questioned. The study is not considered further for the endpoint of reproductive toxicity.

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							shared by all three generations of offspring (F1, F2, and F3). Additionally, only a limited number of phenotypic changes were reported in the F2 and F3 offspring in the glyphosate treated group and most of these were confounded due to the deselection of controls based on obesity. The expected background ranges for findings were not provided; thus, it is not possible to assess whether the findings observed in the glyphosate group animals were outside the normal expected ranges. Some of the reported data appear to be outside the spontaneous ranges reported by other laboratories (i.e., fertility rates and mean litter sizes) and suggest that the study animals may have been of sub standard health. In addition it is not clear what has been actually tested (glyphosate acid, glyphosate salt, glyphosate-based formulation). Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.	
1480	Rappazzo K. M. *et al.*	2019	Maternal residential exposure to specific agricultural pesticide active ingredients and birth defects in a 2003-2005 North Carolina birth cohort.	Birth defects research (2019), Vol. 111, No. 6, pp. 312-323	Exposure to a glyphosate formulation but which not specified.	Background: Previously we observed elevated odds ratios (ORs) for total pesticide exposure and 10 birth defects: three congenital heart defects and structural defects affecting the gastrointestinal, genitourinary and musculoskeletal systems. This analysis	Not relevant by full text: Highly speculative exposure assessment limited to pesticides makes it impossible to adequately assess results. Therefore this study is not relevant.	Due to the limitations and uncertainties in the study, mainly regarding the formulation and the possible impact of co-formulants, the relevance of the results is difficult to assess. However,

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						examines association of those defects with exposure to seven commonly applied pesticide active ingredients. Methods: Cases were live-born singleton infants from the North Carolina Birth Defects Monitoring Program linked to birth records for 2003-2005; noncases served as controls (total n= 304,906). Pesticide active ingredient exposure was assigned using a previously constructed metric based on crops within 500 m of residence, dates of pregnancy, and likely chemical application dates for each pesticide-crop combination. ORs (95% CI) were estimated with logistic regression for categories of exposure compared to unexposed. Models were adjusted for maternal race/ethnicity, age at delivery, education, marital status, and smoking status. Results: Associations varied by birth defect and pesticide combinations. For example, hypospadias was positively associated with exposures to 2,4-D (OR50th to <90th percentile: 1.39 [1.18, 1.64], mepiquat (OR50th to <90th percentile: 1.10 [0.90, 1.34]), paraquat (OR50th to <90th: 1.14 [0.93, 1.39]), and pendimethalin (OR50th to <90th: 1.21 [1.01, 1.44]) but not S-metolachlor (OR50th to 90th: 1.00 [0.81, 1.22]). Whereas atrial septal defects were positively associated with higher levels of exposure to glyphosate, cyhalothrin, S-metolachlor, mepiquat, and pendimethalin (ORs ranged from 1.22 to 1.35 for 50th to <90th exposures, and 1.72 to 2.09 for >90the exposures); associations with paraquat were null or inconsistent (OR 50th to >90th: 1.05 (0.87, 1.27). Conclusion: Our results suggest differing patterns of association for birth defects	Further points for clarification: This study is not relevant to the ongoing evaluation for glyphosate. Comments rendered here are specific for glyphosate, but likely would apply to the other pesticides as well. The most glaring problem with the study is the extreme inadequacy of the exposure assessment. The (presumed) exposure metric is inconsistent with what is known about glyphosate exposure through biomonitoring of individuals not directly involved in a pesticide application and living on farms at the time of application (Acquavella *et al*. 2004, Niemann *et al*. 2015, Solomon 2016). Also, the exposure assessment is based on patterns of applications that happened during years outside of the study period. The exposure metric piles assumptions on top of assumptions with no validation at any stage. Glyphosate biomonitoring data would suggest strongly that few, if any, of the assigned glyphosate exposures would have been validated had the subjects been biomonitored directly. Indirect exposure metrics of the type used in this study should be validated to some	it is noted that cardiac effects were noted also in animal studies with the active substance (see section B.6.8.2).

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						with residential exposure to seven pesticide active ingredients in North Carolina..	degree before they are used in an epidemiologic analysis. Otherwise, they are just speculation, likely with extremely high exposure misclassification ratios. There were also limitations in the analyses in terms of controlling for multiple pesticide and other exposures. Further, the presentation of the results mostly in a graphical format and without p values obscured aspects of the results that would have aided interpretation. Uncertainties about women actually being in their residences or living at the residence of record at the specific times of presumed applications are another source of uncertainty in this study. It seems that all of the non glyphosate pesticides were associated with a birth defect in these analyses. Usually, when every exposure has a positive association with the outcome(s), given that all these pesticides have undergone risk assessments are not considered hazardous to the general public, it suggests the study has a marked problem with false positive findings. Arguments about non differential misclassification are not relevant when the extent of the misclassification is unknown and may be nearly	

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
							complete. Also, there are likely systematic sources of error in this study that are not appreciated by the authors. In conclusion, the results of this study are not reliable for making determinations about glyphosate related birth outcomes. References Acquavella JF, Alexander BH, Mandel JS, *et al*. Glyphosate Biomonitoring for Farmer Applicators and their Families: Results from the Farm Family Exposure Study. Environ Health Perspect 2004; 112:321 326. Niemann L, Sieke C, Pfeil R, Solecki R. A critical review of glyphosate findings in human urine samples and comparison with the exposure of operators and consumers. J Verbr Lebensm 2015; 10:3 12. Solomon K. Glyphosate in the general population and in applicators: a critical review of studies on exposures. Critical Rev Toxicol 2016; 46 Suppl 1:21 27.	
1483	Romano M. A. *et al.*	2012	Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression.	Archives of toxicology (2012), Vol. 86, No. 4, pp. 663-73	No OECD guideline and GLP status. Formulation used. Only one dose tested.	Sexual differentiation in the brain takes place from late gestation to the early postnatal days. This is dependent on the conversion of circulating testosterone into estradiol by the enzyme aromatase. The glyphosate was shown to alter aromatase activity and decrease serum testosterone concentrations. Thus, the aim of this study was to investigate the effect of gestational maternal glyphosate	Not relevant by full text: The test material was a glyphosate based formulation Roundup Transorb which is not the EU representative formulation MON 52276, therefore, the article is not relevant. Furthermore, in the absence of a concurrent control for each of the component of the	The RMS agrees with the applicant´s justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could also be noted that effects on

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						exposure (50 mg/kg, NOAEL for reproductive toxicity) on the reproductive development of male offspring. Sixty-dayold male rat offspring were evaluated for sexual behavior and partner preference; serum testosterone concentrations, estradiol, FSH and LH; the mRNA and protein content of LH and FSH; sperm production and the morphology of the seminiferous epithelium; and the weight of the testes, epididymis and seminal vesicles. The growth, the weight and age at puberty of the animals were also recorded to evaluate the effect of the treatment. The most important findings were increases in sexual partner preference scores and the latency time to the first mount; testosterone and estradiol serum concentrations; the mRNA expression and protein content in the pituitary gland and the serum concentration of LH; sperm production and reserves; and the height of the germinal epithelium of seminiferous tubules. We also observed an early onset of puberty but no effect on the body growth in these animals. These results suggest that maternal exposure to glyphosate disturbed the masculinization process and promoted behavioral changes and histological and endocrine problems in reproductive parameters. These changes associated with the hypersecretion of androgens increased gonadal activity and sperm production.	formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	offspring reproductive toxicity were not reported in standard reproductive toxicity studies using much higher dose levels.
1485	Romano R. M. *et al.*	2010	Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology.	Archives of toxicology (2010), Vol. 84, No. 4, pp. 309-17	No OECD guideline and GLP status. Formulation used.	Glyphosate is a herbicide widely used to kill weeds both in agricultural and non-agricultural landscapes. Its reproductive toxicity is related to the inhibition of a StAR protein and an aromatase enzyme, which causes an *in vitro* reduction in testosterone and	Not relevant by full text: The test material was a glyphosate based formulation Roundup Transorb which is not the EU representative formulation MON 52276, therefore, the article is not relevant.	The RMS agrees with the applicant´s justification. The test substance was a formulation and effects caused by co-formulants cannot be excluded. The study is not considered

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						estradiol synthesis. Studies *in vivo* about this herbicide eVects in prepubertal Wistar rats reproductive development were not performed at this moment. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis. Results showed that the herbicide (1) significantly changed the progression of puberty in a dosedependent manner; (2) reduced the testosterone production, in semineferous tubules' morphology, decreased significantly the epithelium height ($P < 0.001$; control = 85.8 ±2.8 µm; 5 mg/kg = 71.9 ± 5.3 µm; 50 mg/kg = 69.1 ±1.7 µm; 250 mg/kg = 65.2 ± 1.3 µm) and increased the luminal diameter ($P < 0.01$; control = 94.0 ± 5.7 µm; 5 mg/kg = 116.6 ± 6.6 µm; 50 mg/kg = 114.3 ± 3.1 µm; 250 mg/kg = 130.3 ± 4.8 µm); (4) no diVerence in tubular diameter was observed; and (5) relative to the controls, no diVerences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 ±12.9 ng/dL; 5 mg/kg = 108.6 ± 19.6 ng/dL; 50 mg/dL =84.5 ± 12.2 ng/dL; 250 mg/kg = 76.9 ± 14.2 ng/dL). These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor *in vivo*, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.	Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	further for the endpoint of reproductive toxicity. It could also be noted that effects on offspring reproductive toxicity were not reported in standard reproductive toxicity studies using much higher dose levels.
1512	Teleken J. L. *et al.*	2019	Glyphosate-based herbicide exposure during pregnancy and lactation malprograms the male reproductive	Journal of developmental origins of health and disease (2019), Vol. 11, No. 2, pp 146-	No OECD guideline and GLP status. Glyphosate formulation used.	One of the most consumed pesticides in the world is glyphosate, the active ingredient in the herbicide ROUNDUP®. Studies demonstrate that glyphosate can act as an endocrine disruptor and that exposure to this substance at critical	Not relevant by full text: This study described a glyphosate-based herbicide dosed to mice. Roundup Original DI (containing POEA) is not the EU representative	The RMS agrees with the applicant´s justification. The formulation used in the study contains the adjuvant POEA (not permitted for use in EU). Thus, the study is not relevant

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
			morphofunction in F1 offspring.	153		periods in the developmental period may program the fetus to induce reproductive damage in adulthood. Our hypothesis is that maternal exposure to glyphosate during pregnancy and lactation in mice will affect the development of male reproductive organs, impairing male fertility during adult life. Female mice consumed 0.5% glyphosate-ROUNDUP® in their drinking water [glyphosate-based herbicide (GBH) group] or filtered water [control (CTRL) group] from the fourth day of pregnancy until the end of the lactation period. Male F1 offspring were designated, according to their mother's treatment, as CTRL-F1 and GBH-F1. Female mice that drank glyphosate displayed reduced body weight (BW) gain during gestation, but no alterations in litter size. Although GBH male F1 offspring did not exhibit modifications in BW, they demonstrated delayed testicular descent. Furthermore, at PND150, GBH-F1 mice presented a lower number of spermatozoa in the cauda epididymis and reduced epithelial height of the seminiferous epithelium. Notably, intratesticular testosterone concentrations were enhanced in GBH-F1 mice; we show that it is an effect associated with increased plasma and pituitary concentrations of luteinizing hormone. Therefore, data indicate that maternal exposure to glyphosate-ROUNDUP® during pregnancy and lactation may lead to decreased spermatogenesis and disruptions in hypothalamus–pituitary–testicular axis regulation in F1 offspring.	formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	for the EU renewal of active substance. The study is not considered further for the endpoint of reproductive toxicity.
1518	Turkmen R. *et al*.	2019	Prenatal and neonatal exposure to glyphosate-based herbicide reduces the	Kocatepe Veterinary Journal (2019), Vol. 12, No. 2,	No OECD guideline and GLP status. Glyphosate formulation used, few	This study investigated how a glyphosate-based herbicide (GBH) affects the proportional distribution of ovarian follicles that develop from the	Not relevant by full text: Rats gavaged with a glyphosate based herbicide (Knockdown 48 SL; Safa Agriculture Inc.,	The RMS agrees with the applicant´s justification. The test substance was a formulation and effects

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
			primordial to primary follicle transition in the newborn rat ovary: a preliminary study	pp. 168-177	animals and only one dose tested.	18th day of the embryo period (E18) to the 7th postnatal day (PND7) in newborn female rats. A total of 6 pregnant rats that were used in the study were divided into two groups so that there would be 3 pregnant rats in the control group and 3 pregnant rats in the GBH group. Starting from E21 to E18 the pregnant rats in the experimental group were administered at 50 mg/kg/day GBH subcutaneously (s.c.) and the physiological saline was administered as vehicle to the control group. Subsequently, female pups received vehicle or 2 mg/kg GBH from PND1 to PND7. On PND8, all female offspring (neonatal period, 6 newborn female rats from each group) were sacrificed by light ether anesthesia. For the histological examination of the dissected ovaries, the primordial, primary, secondary and preantral follicle numbers were determined using Crossman's modified triple staining method and Periodic Acid-Shiff (PAS) staining methods. The percentage of primordial follicles was significantly higher in the ovaries of female rats in GBH exposed group compare to the control group. However, the percentage of primary, secondary and preantral follicles was lower. Thus, it was observed that prenatal and neonatal GBH exposure decreased the transition of primordial follicle to primary follicle.	Turkey). The formulation tested is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	caused by co-formulants cannot be excluded. The study is not considered further for the endpoint of reproductive toxicity. It could also be noted that effects on ovary were not reported in standard reproductive toxicity studies using much higher dose levels.

Developmental toxicity

Upon review of the titles and abstracts of articles assigned this category, study summaries were requested for the studies listed in the table below to further justify the categorisation of the information. The justification provided by the applicant was reviewed by the RMS and the overall conclusions are presented in the table below.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
1271	Avila-Vazquez M. *et al.*	2018	Environmental exposure to glyphosate and reproductive health impacts in agricultural population of Argentina.	Journal of Environmental Protection (2018), Vol. 9, No. 3, pp. 241-253	The general population was exposed to multiple environmental factors	"Argentina annually utilizes 240,000 tons of glyphosate in industrial agriculture and a change in the profile of morbidity is perceived for physicians of agricultural areas; now reproductive disorders seem to prevail. The objective of this study is to determine concurrence of glyphosate exposure and reproductive disorders in a typical argentine agricultural town (Monte Maíz). An ecological study was developed with an environmental analysis of pollution sources including measurements of glyphosate and other pesticides and a cross-sectional study of spontaneous abortions and congenital abnormalities prevalence. Glyphosate was detected in soil and grain dust and was found to be at an even higher concentration in the village soil than in the rural area; 650 tonnes of glyphosate are used annually in the region and manipulated inner town contaminating the soil and dust in suspension of the town creating an burden of environmental exposure to glyphosate of 79 kg per person per year. We do not find other relevant sources of pollution. The spontaneous abortion and congenital abnormalities rates are three and two times higher than the national average reported by the national health (10% vs. 3% and 3% - 4.3% vs 1.4% respectively). Our study verified high environmental exposure to glyphosate in association with increased frequencies of reproductive disorders (spontaneous abortion and congenital	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate because the general population followed was exposed to multiple environmental factors making it impossible to establish a causal relationship between exposure to glyphosate and reproductive disorders. Further points for clarification: This study is not relevant to the ongoing assessment of glyphosate for several reasons. First, the exposure assessment is ecologic; that is, exposures are not known on an individual level (the basis for the ecologic fallacy) for the population being studied and the subpopulation of those who experienced spontaneous abortions and birth defects. One cannot conclude from environmental measurements that an affected individual had an appreciable exposure during the relevant biologic time window. Second, if one wished to compare local and national rates for reproductive outcomes, standard practice would be to assess outcomes similarly in the small village and nationally. That is obviously not the case in this article. Also, consider that previous research shows that 10% to 20% of pregnancies result in	The RMS agrees with the applicant´s justification, also considering that the study was carried out in a country outside EU.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						abnormalities) in argentine agricultural village but is unable to make assertions cause-effect. Further studies are required with designs for such purposes."	spontaneous abortions, perhaps up to 25% in some studies. The 10% reported in this study for the agricultural village is at the low end of the expected range. The national estimate of approximately 1/3rd of the prevalence in the small village would be markedly below the low end of the range of previous research. It is likely not a valid estimate for the national population. The literature, therefore, strongly suggests that the local and national comparison is not valid. Third, the analysis within the village that compared the higher versus lesser-exposed areas showed an odds ratio of 1.29 (95% CI 0.71, 2.34). The authors also noted that the p value was not significant. Indeed, given the lower bound of the confidence interval, it was not even close to being significant. So, within the village, spontaneous abortion did not correspond to presumed higher exposure, though the authors concluded otherwise. The investigators misinterpreted the non significant p value as indicating that bias could not be ruled out. That is true. But, of course, p values and confidence intervals are calculated under the assumption of no bias and represent random error, not systematic error (bias), in statistical analyses. The fact that systematic error seems not to have been rigorously evaluated in this study is a separate issue. In conclusion, this study has severe limitations. The exposure measure is ecologic, not individual. The comparison of local versus national	

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
							rates is likely based on different methodologies for identifying outcomes. It is noteworthy that the proportions of adverse outcomes at local and national levels are at the low end of those reported in the literature especially the national estimate. Within the agricultural area, there was no apparent association between presumed exposure and spontaneous abortion. Taken together, these limitations make the results uninterpretable with respect to glyphosate exposure.	
1452	Meyer-Monath M. *et al.*	2014	Development of a multi-residue method in a fetal matrix: analysis of meconium	Analytical and Bioanalytical Chemistry (2014), Vol. 406, No. 30, pp. 7785-7797	Analytical method development. No data shown on glyphosate in actual meconium samples.	Meconium is the earliest stool of newborns. It is a complex matrix that reflects the degree of fetal exposure to environmental pollutants. To investigate exposure to xenobiotics, an analytical method was developed to identify and quantify some pesticides and their metabolites and BTEX metabolites in meconium. Samples were prepared by two liquid–solid extractions and purified twice using SPE cartridges, followed by analysis with liquid chromatography coupled with tandem mass spectrometry. SPE cartridges (polymeric phase with hydrophilic and hydrophobic interactions, ion exchange, mixed mode) were tested and matrix effects were evaluated to determine purification performance. The quantification limits in meconium of this multi-residue method were in the range of 30 ng g−1. The analytical method was applied to “real” meconium samples. Some target analytes were determined in most samples.	Not relevant by full text: This is primarily an analytical method paper for determination of multiple analytes (including glyphosate) in meconium. Actual meconium samples were analyzed. Minimal details of results provided, and no detections of glyphosate reported, therefore the report is not relevant.	The RMS agrees that the study is not considered relevant for this review.
1512	Teleken J. L. *et al.*	2019	Glyphosate-based herbicide exposure during pregnancy and lactation malprograms the	Journal of developmental origins of health and disease (2019), Vol. 11,	No OECD guideline and GLP status. Glyphosate formulation used.	One of the most consumed pesticides in the world is glyphosate, the active ingredient in the herbicide ROUNDUP®. Studies demonstrate that glyphosate can act as an endocrine disruptor and that	Not relevant by full text: This study described a glyphosate-based herbicide dosed to mice. Roundup Original DI (containing POEA) is not the EU representative	The RMS agrees with the applicant´s justification. The formulation used in the study contains the adjuvant POEA (not

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
			male reproductive morphofunction in F1 offspring.	No. 2, pp 146-153		exposure to this substance at critical periods in the developmental period may program the fetus to induce reproductive damage in adulthood. Our hypothesis is that maternal exposure to glyphosate during pregnancy and lactation in mice will affect the development of male reproductive organs, impairing male fertility during adult life. Female mice consumed 0.5% glyphosate-ROUNDUP® in their drinking water [glyphosate-based herbicide (GBH) group] or filtered water [control (CTRL) group] from the fourth day of pregnancy until the end of the lactation period. Male F1 offspring were designated, according to their mother's treatment, as CTRL-F1 and GBH-F1. Female mice that drank glyphosate displayed reduced body weight (BW) gain during gestation, but no alterations in litter size. Although GBH male F1 offspring did not exhibit modifications in BW, they demonstrated delayed testicular descent. Furthermore, at PND150, GBH-F1 mice presented a lower number of spermatozoa in the cauda epididymis and reduced epithelial height of the seminiferous epithelium. Notably, intratesticular testosterone concentrations were enhanced in GBH-F1 mice; we show that it is an effect associated with increased plasma and pituitary concentrations of luteinizing hormone. Therefore, data indicate that maternal exposure to glyphosate-ROUNDUP® during pregnancy and lactation may lead to decreased spermatogenesis and disruptions in hypothalamus–pituitary–testicular axis regulation in F1 offspring.	formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.	permitted for use in EU). Thus, the study is not relevant for the EU renewal of active substance.
	Alarcon R. *et al.*	2020	Neonatal exposure to a glyphosate-based herbicide alters the uterine	Environmental pollution, (2020) Vol. 265, No. Pt B, Art. No.	No OECD guideline and GLP status. Glyphosate formulation used,	The exposure to endocrine-disrupting compounds (EDCs), such as glyphosate-based herbicides (GBHs),during early life might alter female fertility. The aim of the	Roundup FULL II® (Argos SRL, Santa Fe, Argentina), a liquid water-soluble formulation containing 54 g of glyphosate in 100 mL of	The study is considered of limited value since the test substance was a formulation and effects

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
			differentiation of prepubertal ewe lambs.	114874	*s.c.* administration and only one dose was tested.	present study was to evaluate the effects of neonatal exposure to a GBH on sheep uterine development. To achieve this, Friesian ewe lambs were exposed to GBH (2 mg/kg of body weight/day; n ¼ 12) or vehicle (controls; n ¼ 10) through s.c. injections, from postnatal day (PND) 1 to PND14; on PND45, the uteri were obtained to evaluate histomorphological and molecular parameters. Morphological parameters were determined by picrosiriushematoxylin staining. Protein expression of Ki67 (as a cell proliferation marker), p27, and molecules involved in uterine organogenetic differentiation was measured by immunohistochemistry. We also determined the mRNA expression of the IGF molecular pathway by RT-PCR. Although histomorphology was not modified, the uteri of GBH-exposed ewe lambs showed lower cell proliferation, together with higher p27 protein expression. In addition, the uteri of GBH-exposed ewe lambs showed increased gene expression of insulin-like growth factor binding protein 3 (IGFBP-3), decreased expression of ERa in the luminal (LE) and glandular (GE) epithelia and in the subepithelial stroma (SS), and lower PR expression in the LE but higher in the GE and SS. In addition, GBH treatment decreased the uterine expression of Wnt5a in the GE, of Wnt7a in the SS, of b-catenin in the LE and GE, of Hoxa10 in the SS, and of Foxa2 in the GE as compared with controls. In conclusion, neonatal exposure to GBH decreased cell proliferation and altered the expression of molecules that control proliferation and development in the uterus. All these changes might have adverse consequences on uterine differentiation and functionality, affecting the female reproductive health of sheep.	commercial formulation is not the EU representative formulation for the EU glyphosate renewal and therefore not relevant to the EU glyphosate renewal. In addition, direct injection into the neck of pregnant ewes with formulated product containing surfactant is not relevant to real life exposure scenarios. Environmental fate, metabolism and pharmacokinetics for glyphosate active ingredient versus surfactants are very different. Given the direct systemic exposure, these data are considered irrelevant to livestock and human health risk assessments.	caused by co-formulants cannot be excluded. Moreover, administration was via s c. injection, only one dose was tested and it is noted that lamb is an unusual test species in toxiclological studies.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						GBH may be responsible for uterine subfertility, acting as an EDC.		
66	Gomez A. L. *et al.*	2020	Exposure to a Glyphosate-based Herbicide Alters the Expression of Key Regulators of Mammary Gland Development on Pre-pubertal Male Rats.	Toxicology, (2020) Vol. 439, Art No. 152477	No OECD guideline and GLP status. Glyphosate formulation used and only two doses were tested without any dose response.	We previously reported that exposure during gestation and lactation to a low dose of glyphosate-based herbicide (GBH) reduced the area and perimeter of male offspring mammary gland at postnatal day 60 (PND60), whereas a higher dose increased the longitudinal growth of the gland. Here, our aim was to assess whether perinatal exposure to GBH exhibits endocrine disruptive action in male mammary gland at an early time point (prepuberty), which could be related to the changes observed after puberty. We also wanted to explore whether an early evaluation of the male rat mammary gland is appropriate to assess exposure to potential endocrine disrupting chemicals (EDCs). Pregnant rats were orally exposed, through the diet, to vehicle (saline solution), 3.5 or 350 mg/kg/day of GBH from gestational day 9 until weaning. At PND21, the male offspring were euthanized, and mammary gland samples were collected. The histology and proliferation index of the mammary glands were evaluated, and the mRNA expression of estrogen (ESR1) and androgen (AR) receptors, cyclin D1 (Ccnd1), amphiregulin (Areg), insulin-like growth factor 1 (IGF1), epidermal growth factor receptor (EGFR) and IGF1 receptor (IGF1R) were assessed. Moreover, the phosphorylated-Erk1/2 (p-ERK1/2) protein expression was determined. No differences were observed in mammary epithelial structures and AR expression between experimental groups; however, the proliferation index was reduced in GBH3.5-exposed males. This result was associated with decreased ESR1, Ccnd1, Areg, IGF1, EGFR and IGF1R mRNA	*In vivo* study on pre- and post-natal effects of Glyphosate-based herbicide administered to Wistar female rat at 3 5 and 350 mg/kg/day (8-10 females rat/group). Glyphosate-based herbicide (Glyphosate 66.2% - potassium salt, acid equivalent 54%) was tested, which is not the EU representative formulation and thus article is not relevant for the EU glyphosate renewal.	The study is considered of limited value since the test substance was a formulation and effects caused by co-formulants cannot be excluded. Moreover, only two doses were tested without any dose response.

No	Author(s)	Year	Title	Source	Deviation(s) from regulatory guidelines for standard toxicity studies	Abstract	Reason by applicant for not including publication in dossier	Comment(s)
						expressions, as well as reduced p-Erk1/ 2 protein expression in these animals. ESR1, Ccnd1, IGF1R and EGFR expressions were also reduced in GBH350- exposed males. In conclusion, the mammary gland development of pre-pubertal male rats is affected by perinatal exposure to GBH. Although further studies are still needed to understand the molecular mechanisms involved in GBH350 exposure, the present results may explain the alterations observed in mammary gland growth of postpubertal males exposed to low doses of GBH. Our results also suggest that early evaluation of the male rat mammary gland is useful in assessing exposure to potential EDCs. However, analysis of EDCs effects at later time points should not be excluded.		

Study summaries of publications classified by the applicant as "non-relevant after detailed assessment of full-text article"

The study summaries below were submitted in response to a request from the RMS since more information was considered necessary to assess the justification for the classification as "non-relevant".
The summaries below are provided in alphabetical order and presented by technical discipline.

Data point	CA 9
Author	Abarikwu S. O. *et al.*
Year	2015
Title	Combined effects of repeated administration of Bretmont Wipeout (glyphosate) and Ultrazin (atrazine) on testosterone, oxidative stress and sperm quality of Wistar rats
Document source	Toxicology Mechanisms and Methods (2015), Vol. 25, No. 1, pp. 70-80
Short description of literature article	The potential toxicity resulting from the possible interactions of the herbicide Bretmont Wipeout (glyphosate, GLY) (as commercialized in Nigeria) is not completely known. This report evaluated reproductive and hepato toxicity in rats exposed to GLY. Six weeks old male rats were exposed by gavage three times per week to GLY (5 mg/kg) alone or in combination with atrazine (ATZ) (5 mg/kg GLY + 12.5 mg/kg ATZ) or vehicle (corn oil), for 52 days. The authors concluded that some of the herbicides currently used in Nigeria by farmers are capable of inducing hepato and testicular toxicity in rats at low doses. Moreover, there were antagonistic interactions between the two toxicants on the toxicity endpoints investigated in this study, and these effects were due to the active ingredients of both herbicides in the commercial formulations.
Short description of findings	This study involved single dose effects of both GLY and ATZ alone or in combination. GLY impaired sperm quality. Testosterone level, sperm motility, sperm counts, live/dead ratio and the weight of the epididymis were lower in the GLY group compared to the ATZ group by 57%, 33%, 20%, 22% and 41%, and higher by 109%, 76.7%, 39.6%, 32.3%, and 100%, respectively, in the combined exposure group (ATZ + GLY) compared to the GLY group. Oxidative stress and histopathological changes were also noticeable in the liver but not in the testis of GLY treated animals, and the observed effects were more remarkable in the GLY group than the ATZ or the combined exposure group. The combined effects of the active ingredients on testosterone level, sperm count and hepatic malondialdehyde levels were also similar as when the commercial formulations were used. The authors suggested that the effect of ATZ on the investigated toxicity endpoints was milder than the effect of GLY. On the combination of both herbicides, ATZ reduced the effect of GLY resulting in an overall mild effect on the oxidative stress parameters of the liver and testis and sperm quality variables. The authors considered that an antagonistic interaction could potentially exist when commercial formulations of ATZ and GLY are administered together. However, the authors indicated that the observed combined effects were limited to the study's experimental conditions, which might not reveal the molecular mechanism of the interaction leading to the antagonism.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation provided to Wistar rats via oral gavage in corn oil. The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study is a 450 g/L product differing in components to the EU representative formulation MON 52276. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Alarcon R. *et al.*
Year	2019
Title	Neonatal exposure to a glyphosate based herbicide alters the histofunctional differentiation of the ovaries and uterus in lambs
Document source	Molecular and Cellular Endocrinology (2019), Vol. 482, pp. 45-56
Short description of literature article	The authors hypothesized that postnatal exposure of lambs to a glyphosate based herbicide (GBH) in a critical phase of development (first postnatal days) could alter ovarian and uterine differentiation. Therefore, the study aimed to compare the effect of oral and subcutaneous neonatal exposure to a low dose of GBH on the ovaries and uteri of prepubertal lambs one month after exposure ended. To this end, ewe lambs were exposed to a subcutaneous (n = 5) or an oral (n = 5) environmentally relevant dose of GBH (2 mg/kg/day) or to vehicle (controls, n = 12), from postnatal day (PND) 1 to PND14. These doses were prepared by dissolution in saline solution of the commercial glyphosate formulation Roundup Full II (Argos SRL, Argentina), a liquid water soluble formulation containing 54 g of glyphosate in 100 mL. Serum glyphosate and aminomethylphosphonic acid (AMPA) concentrations were measured on PND15 and PND45. The ovaries and uterus were obtained and weighed on PND45. Ovarian follicular dynamics and uterine morphological features were determined by picrosirius hematoxylin staining. The proliferation marker Ki67 was evaluated by immunohistochemistry in ovarian and uterine samples.
Short description of findings	The authors detected glyphosate but not AMPA in the serum of exposed lambs at the end of the exposure period (PND15). Glyphosate serum levels at PND15 were similar regardless of the administration route. On the other hand, neither glyphosate nor AMPA was detected on PND45. Control lambs were negative for glyphosate and AMPA on PND15 and PND45. Moreover, GBH exposure did not affect ovarian or uterine weight. However, on PND45, the ovaries of subcutaneous or orally GBH exposed lambs showed altered follicular dynamics, increased proliferation of granulosa and theca cells, and decreased mRNA expression of the follicle stimulating hormone receptor (FSHR) and growth and differentiation factor 9 (GDF9), whereas their uterus showed decreased cell proliferation but no alterations in the histomorphology or gene expression. The authors concluded that GBH exposure altered the ovarian follicular dynamics and gene expression, and the proliferative activity of the ovaries and uterus of lambs. All the adverse effects found in the ovaries and uterus of both GBH exposed groups were similar, independently of the administration route. Nevertheless, the authors could not attribute the observed effects to glyphosate, the co formulants or the mixture.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Roundup Full II, Argos SRL, Santa Fe, Argentina; 54 g/100 mL glyphosate) is not the EU representative formulation, therefore the article is not relevant for glyphosate EU renewal. The authors state they could not attribute any observed adverse effects to glyphosate, the co formulants or the mixture.

Data point	CA 9
Author	Altamirano G. A. *et al.*
Year	2018
Title	Postnatal exposure to a glyphosate based herbicide modifies mammary gland growth and development in Wistar male rats
Document source	Food and Chemical Toxicology (2018), Vol. 118, pp. 111-118
Short description of literature article	This study aimed to evaluate whether early postnatal exposure to a glyphosate based herbicide (GBH) modifies mammary gland growth and development in pre and post pubertal male rats. From postnatal day 1 (PND1) to PND7, male rats were injected subcutaneously every 48 h with either saline solution (vehicle) or 2 mg GBH/kg body weight. The composition of the glyphosate commercial formulation used (Roundup FULL II) was a liquid water soluble preparation containing glyphosate potassium salt (54 g of glyphosate per 100 mL of GBH), as its active ingredient, adjuvants and inert ingredients. On PND21 and PND60, mammary gland and blood samples were collected. Estradiol (E2) and testosterone (T) serum levels, mammary gland histology, collagen fibre organization, mast cell infiltration, proliferation index, and estrogen (ESR1) and androgen receptor (AR) expression levels were evaluated. The results showed that a postnatal subacute treatment with GBH induces endocrine disrupting effects in the male mammary gland *in vivo*, altering its normal development.
Short description of findings	At PND21, GBH exposed male rats exhibited greater development of the mammary gland with increased stromal collagen organization and terminal end buds (TEBs) compared to control rats. At PND60, the number of TEBs remained high and was accompanied by an increase in mast cell infiltration, proliferation and ESR1 expression in GBH exposed male rats. In contrast, no effects were observed in E2 and T serum levels and AR expression in both days studied. The authors concluded that exposure to GBH during the first week of life has an endocrine disruption effect in the male mammary gland *in vivo*. Exposure to GBH could accelerate the development of the mammary gland tree in pre pubertal rats and the persistence of highly proliferative structures in post pubertal animals together with high ESR1 expression. This was shown by an increment of the mammary total area and perimeter in GBH exposed rats at PND21. The authors noted that all these observed effects were due to the exposure to the complex mixture of the active principle (glyphosate) accompanied by several adjuvants. Additional studies are needed to demonstrate whether these effects on male mammary gland development are caused by glyphosate alone or its combination with adjuvants.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Roundup FULL II, potassium salt; 54% a.e.). The formulation tested contains potassium salts and co formulants not directly comparable to the EU representative formulation MON 52276, therefore the article is not relevant for the glyphosate EU renewal. The authors state that additional studies are needed to demonstrate whether effects on male mammary glands are a potential result of glyphosate alone or in combination with adjuvants. The subcutaneous dosing to developing rats does not directly simulate anticipated operator or consumer exposure routes. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Anifandis G. *et al.*
Year	2017
Title	The *in vitro* impact of the herbicide roundup on human sperm motility and sperm mitochondria
Document source	Toxics (2017), Vol. 6, No. 1, pp. 2
Short description of literature article	Herbicides have been hypothesized to affect sperm parameters. The most common method of exposure to herbicides is through spraying or diet. Therefore, this study investigated the effect of direct exposure of human sperm cells to 1 mg/L of the herbicide Roundup on sperm motility and mitochondrial integrity. Sperm samples from 66 healthy men who were seeking semen analysis were investigated after their written informed consent. Semen analysis was performed according to the 2010 World Health Organization guidelines. Mitochondrial integrity was assessed through mitochondrial staining using a mitochondria specific dye, which is incorporated exclusively into functionally active mitochondria. The study results indicated that the direct exposure of semen samples to the active constituent of the herbicide Roundup at the low concentration of 1 mg/L had adverse effects on sperm motility, which might be related to the observed reduction in mitochondrial staining.
Short description of findings	A quantity of 1 mg/L of Roundup was found to exert a deleterious effect on sperm's progressive motility after 1 hour of incubation (mean difference between treated and control samples = 11.2%; 46.42 ± 16.19% versus 35.26 ± 15.21%) in comparison with the effect observed after 3 hours of exposure (mean difference = 6.33%; 30.53 ± 11.67% versus 36.86 ± 13.42%, $p < 0.05$). Moreover, the evaluation of the relative fluorescence intensity per unit area showed that the incorporation of the mitochondrial dye in mitochondria of the mid piece region of Roundup treated spermatozoa was significantly reduced compared to controls (0.66 ± 0.49 versus 1.21 ± 0.96, $p < 0.05$), at the first hour of incubation, indicating mitochondrial dysfunction by Roundup. The authors concluded that the direct *in vitro* impact of Roundup on human sperm motility was demonstrated for the first time, possibly via mitochondrial deregulation. The authors suggested that Roundup at 1 mg/L could cause male sub fertility, indicating that more studies are needed to ascertain the biochemical mechanism of action of Roundup on sperm motility and mitochondrial impairment.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested *in vitro* (Roundup, not characterized). Participants in the study were apparently not screened for any previous exposures or environmental factors prior to the study and the specific motivations for their involvement in the study was not reported. No apparent evaluation of the quality or motility of the sperm was assessed prior to the study and this evaluation is important to determine if the participants had underlying health conditions that affected semen quality or why they were seeking semen analysis potentially due to infertility or other pre-existing health conditions. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Avdatek F. *et al.*
Year	2018
Title	Ameliorative effect of resveratrol on testicular oxidative stress, spermatological parameters and DNA damage in glyphosate based herbicide exposed rats.
Document source	Andrologia (2018), Vol. 50, No. 7, pp. e13036
Short description of literature article	The objective of this study was to investigate the protective role of resveratrol (RES) in motility, abnormal sperm rate, plasma membrane integrity, oxidative stress, and DNA damage of rats spermatozoa induced by glyphosate (GLF). Therefore, the authors assessed the reproductive impacts of being exposed to GLF and the protective impacts of RES in 28 Wistar male rats, equally separated into four groups. Control group were fed a normal diet without GLF or RES, group II received normal feed containing 20 mg/kg/daily RES, group III received normal feed containing 375 mg/kg/daily GLF, and group IV received normal feed containing 375 mg/kg/daily GLF + 20 mg/kg/daily RES. Glyphosate (Knockdown 48 SL) was obtained from Hektas company (Kocaeli, Turkey). The study findings indicated that RES protects spermatological parameters and DNA damage, decreases GLF induced lipid peroxidation, improves the antioxidant defence mechanism and regenerates tissue damage in the testis of rats.
Short description of findings	GLF administration significantly decreased sperm motility, sperm plasma membrane integrity, glutathione levels, and superoxide dismutase in the testicular tissue of rats in comparison with the control group ($p < 0.05$). On the other hand, abnormal sperm rate, malondialdehyde levels, and DNA damage were detected to be significantly higher in the group treated with GLF compared to the control group ($p < 0.05$). Moreover, a decrease in sperm concentration and degeneration of Sertoli cells have been detected in the testis in the GLF and GLF + RES groups. The authors concluded that RES showed a protective effect and antioxidant activity on the germ cells of the rat testes against adverse effects induced by GLF.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Glyphosate based formulation tested (Knockdown 48 SL) contains a different overall composition, which is not comparable to the representative EU formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Avdatek F. *et al.*
Year	2018
Title	Protective effect of N acetylcysteine on testicular oxidative damage, spermatological parameters and DNA damage in glyphosate based herbicide exposed rats.
Document source	Kocatepe Veterinary Journal (2018), Vol. 11, No. 3, pp. 292-300
Short description of literature article	This study aimed was to examine the protective effect of N acetylcysteine (NAC) on testicular oxidative damage, spermatological parameters, and DNA damage caused by glyphosate (GLF) in rats. In total, 28 Wistar Albino male rats were evaluated by being separated into four groups in an equal way. Rats in group I, which represented the control group, were fed normal diet without GLF or NAC, group II received normal feed containing 160 mg/kg/daily NAC, group III received normal feed containing 375 mg/kg/daily GLF, and group IV received normal feed containing 160 mg/kg/daily NAC + 375 mg/kg/daily GLF. The authors used the commercial preparation Knockdown 48 SL (Hektas, Turkey) as the GLF source. The differences between the groups concerning to all the sperm properties, histological results, and biochemical parameters were assessed by the one way analysis of variance (ANOVA) and post hoc Duncan test. The study results indicated that NAC reduced lipid peroxidation caused by GLF, improved the antioxidant defense mechanism and regenerated tissue damage in rats' testis.
Short description of findings	In this study, GLF caused a reduction in sperm motility, plasma membrane integrity of spermatozoa, and an increase in abnormal sperm rate in the rats' testicular tissue. In the group administered with GLF, the malondialdehyde content of the testis significantly increased ($p < 0.05$) while the testicular superoxide dismutase activity significantly reduced ($p < 0.05$), when compared to the control group. The authors also reported that GLF damaged sperm DNA and caused a more significant DNA damage in comparison with the control group ($p < 0.05$). Moreover, in histopathological terms, the authors detected a decrease in sperm concentration and degeneration of Sertoli cells in the testicular tissue in the GLF group.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Glyphosate based formulation tested (Knockdown 48 SL) contains a different overall composition, which is not comparable to the representative EU formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Avila-Vazquez M. *et al.*
Year	2018
Title	Environmental exposure to glyphosate and reproductive health impacts in agricultural population of Argentina
Document source	Journal of Environmental Protection (2018), Vol. 9, No. 3, pp. 241-253
Short description of literature article	The authors conducted an ecologic analysis of environmental glyphosate (and other pesticides) concentrations and the cross sectional proportion of spontaneous abortions and live births with congenital abnormalities in a small village compared with national figures. They also presented an analysis of spontaneous abortions within the village area for areas of presumed higher versus lower exposure.
Short description of findings	The authors concluded that their study found high environmental glyphosate concentrations in an area with elevated frequencies of spontaneous abortions and congenital abnormalities. They concluded, however, that the ecologic nature of the design (viz., no knowledge of exposure status for the mothers with adverse outcomes) precluded conclusions about cause and effect.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate because the general population followed was exposed to multiple environmental factors making it impossible to establish a causal relationship between exposure to glyphosate and reproductive disorders. **Further points for clarification:** This study is not relevant to the ongoing assessment of glyphosate for several reasons. First, the exposure assessment is ecologic; that is, exposures are not known on an individual level (the basis for the ecologic fallacy) for the population being studied and the subpopulation of those who experienced spontaneous abortions and birth defects. One cannot conclude from environmental measurements that an affected individual had an appreciable exposure during the relevant biologic time window. Second, if one wished to compare local and national rates for reproductive outcomes, standard practice would be to assess outcomes similarly in the small village and nationally. That is obviously not the case in this article. Also, consider that previous research shows that 10% to 20% of pregnancies result in spontaneous abortions, perhaps up to 25% in some studies. The 10% reported in this study for the agricultural village is at the low end of the expected range. The national estimate of approximately 1/3rd of the prevalence in the small village would be markedly below the low end of the range of previous research. It is likely not a valid estimate for the national population. The literature, therefore, strongly suggests that the local and national comparison is not valid. Third, the analysis within the village that compared the higher versus lesser-exposed areas showed an odds ratio of 1.29 (95% CI 0.71, 2.34). The authors also noted that the p value was not significant. Indeed, given the lower bound of the confidence interval, it was not even close to being significant. So, within the village, spontaneous abortion did not correspond to presumed higher exposure, though the authors concluded otherwise. The investigators misinterpreted the non significant p value as indicating that bias could not be ruled out. That is true. But, of course, p values and confidence intervals are calculated under the assumption of no bias and represent random error, not systematic error (bias), in statistical analyses. The fact that systematic error seems not to have been rigorously evaluated in this study is a separate issue. In conclusion, this study has severe limitations. The exposure measure is ecologic, not individual. The comparison of local versus national rates is likely based on different methodologies for identifying outcomes. It is noteworthy that the proportions of adverse outcomes at local and national levels are at the low end of those reported in the literature especially the national estimate. Within the

agricultural area, there was no apparent association between presumed exposure and spontaneous abortion. Taken together, these limitations make the results uninterpretable with respect to glyphosate exposure.

Data point	CA 9
Author	Bali Y. A. *et al.*
Year	2019
Title	Learning and memory impairments associated to acetylcholinesterase inhibition and oxidative stress following glyphosate based herbicide exposure in mice
Document source	Toxicology (2019), Vol. 415, pp. 18-25
Short description of literature article	The ability of glyphosate (Gly) to cross the blood brain barrier may have adverse effects on the structure and various functions of the nervous system. In this study, the authors investigated the learning and memory functions through a set of behavioural tests in mice exposed to a glyphosate based herbicide (GBH) and assessed its influence on the activity of acetylcholinesterase (AChE) and oxidation/antioxidation homeostasis in mice brains. Male Swiss mice were subjected either to orally gavages by tap water, 250 or 500 mg/kg/day of GBH dissolved in tap water at a concentration of 50 g/L of Gly under acute (unique administration), subchronic (6 weeks), or chronic (12 weeks) treatment. The used GBH was Roundup (Gly concentration 360 g/L in the form of isopropylamine salt) in the liquid commercial form supplied by Monsanto (USA). The integrity of learning and memory was assessed using a specific behavioural test battery: Novel object recognition, Y-maze and passive avoidance tasks. The activities of AChE and anti-oxidant enzymes, namely superoxide dismutase (SOD) and peroxidase (PO), were also evaluated. The study's results indicated that GBH induced numerous cognitive abnormalities referred to different forms of memory likely associated with significant inhibition of AChE activity and oxidative stress induction.
Short description of findings	The authors reported that, unlike acute treatment, both subchronic and chronic exposure to GBH decreased discrimination index and the step-through-latency compared to control groups, which indicated recognition and retention memory impairments, respectively. In contrast, only chronic exposure at both 250 and 500 mg/kg affected working memory manifested by decreased spontaneous alternation. Furthermore, the results showed also a significant decrease in the specific activities of AChE, SOD and PO within the brain of treated mice following both subchronic and chronic exposures. The authors concluded that this study demonstrated the role of central AChE inhibition and oxidative stress as potential mechanisms of GBH to induce learning and memory impairments in mice. The authors also stated that this report might contribute to the clarification of the etiological role of pesticides in human brain diseases.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Roundup herbicide (glyphosate concentration 360 g/L IPA salt, Monsanto) contains a surfactant not present in the representative glyphosate formulation MON 52276 used in the EU renewal process. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Bhardwaj J. K. *et al.*
Year	2019
Title	Effective attenuation of glyphosate induced oxidative stress and granulosa cell apoptosis by vitamins C and E in caprines.
Document source	Molecular Reproduction and Development (2019), Vol. 86, No. 1, pp. 42-52
Short description of literature article	Pesticides are known to cause a wide range of reproductive problems that have degenerative effects on mammalian fertility. Glyphosate (GLP) is known to be a potent mammalian toxicant. The present study aimed at assessing the GLP-induced (0.1, 2.0, and 4.0 mg/mL) granulosa cells toxicity and evaluating the mitigating effects of vitamins C and E (0.5 mM and 1.0 mM) in healthy caprine antral follicles, cultured *in vitro* in a dose and time dependent manner (24, 48, and 72 h). Commercially available Roundup formulation with a purity of 41% (Monsanto India Limited, India) was used to prepare the studied concentrations of GLP. Various cytotoxic and genotoxic analysis were performed, namely, classic histology, ethidium bromide and acridine orange (EB/AO) differential staining, oxidative stress parameters, and antioxidant enzymatic activity. Vitamins C and E supplementation decreased oxidative stress-mediated granulosa cells (GCs) apoptosis, suggesting its efficiency to diminish GLP mediated GCs cytotoxicity and thereby, preventing associated fertility disorders.
Short description of findings	The histomorphological analysis and EB/AO staining showed an increased incidence of apoptotic features within GCs of GLP-treated groups at 4.0 mg/mL GLP, as compared with control (no treatment) and at 24-, 48-, and 72-h exposure duration. The highest apoptotic frequency was observed at 4.0 mg/mL GLP after 72-h exposure duration in comparison with the control. GLP exposure also led to a significant decline in the activity of antioxidant enzymes, namely, superoxide dismutase, catalase, and glutathione-S-transferase along with enhanced lipid peroxidation and reduced ferric reducing antioxidant power activity in a dose- and time-dependent manner.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Glyphosate based herbicide tested with *in vitro* test system. The 41% Indian Roundup formulation testing is not comparable in composition and co formulants to the EU representative formulation MON 52276. Therefore, the article is not considered relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Cassault Meyer E. *et al.*
Year	2014
Title	An acute exposure to glyphosate based herbicide alters aromatase levels in testis and sperm nuclear quality
Document source	Environmental Toxicology and Pharmacology (2014), Vol. 38, No. 1, pp. 131-40
Short description of literature article	Roundup is the major pesticide used in agriculture worldwide; it is a glyphosate based herbicide (GBH). However, studies about the impact of acute and transitory exposure to GBH on reproductive functions are scarce. This study investigated the reproductive effects of acute exposure to GBH and the possible mechanism implicated in the regulation of the androgen/estrogen balance (estrogen receptors ESR1 and ESR2, androgen receptor, Gper1 and P450 arom) at a molecular level in testis and epididymal sperm. The source of the GBH was the commercial formulation Roundup Grand Travaux Plus (Monsanto) composed of 450 g/L glyphosate, 607 g/L isopropylamine salt and adjuvants. GBH molecular effects were studied following acute exposure (0.5% dose) of fifteen 60-day-old male rats during 8 days. Endocrine (aromatase, estrogen and androgen receptors, Gper1 in testicular and sperm mRNAs) and testicular functions (organ weights and sperm parameters) were monitored immediately after the treatment (day 68), after one cycle of spermiogenesis (day 87), and after one cycle of spermatogenesis (day 122). The authors also investigated molecular markers of the blood testis barrier (BTB) integrity (connexin-43, occludin, claudin, and N-cadherin), Sertoli cell junctional proteins, being important for spermatogenesis.
Short description of findings	The authors found no significant changes in the absolute or relative weights of testes or epididymis. Among molecular markers implicated in the androgen/estrogen balance, the major disruption was an increase of the P450 arom transcript levels at least by 50% in treated rats at all times, as well as the aromatase protein. The authors also reported a similar increase of Gper1 expression at day 122 and a light modification of BTB markers. A rise of abnormal sperm morphology and a decrease of the expression of protamine 1 and histone 1 testicular in epididymal sperm were observed despite a normal sperm concentration and motility. The authors concluded that acute exposure of GBH causes molecular changes in the reproductive function, and hypothesized that the repetition of successive exposures of GBH at sub-agricultural doses could alter the mammalian reproductive system over the long term.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate because a glyphosate formulation (Roundup Grand Travaux Plus, composed of 450 g/L glyphosate, 607 g/L isopropylamine salt and adjuvants such as polyoxyethylamine [POEA]) was tested instead of the representative 360 g/L glyphosate formulation MON 52276. In addition, the data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains polyoxyethylene tallow amine (POEA), which is not permitted for use in formulated herbicidal products in the EU.

Data point	CA 9
Author	Cattani D. *et al*.
Year	2017
Title	Developmental exposure to glyphosate based herbicide and depressive like behavior in adult offspring: implication of glutamate excitotoxicity and oxidative stress
Document source	Toxicology (2017), Vol. 387, pp. 67-80
Short description of literature article	This follow up study investigated if the effects on neurochemical and behavioural parameters of maternal subchronic exposure to glyphosate based herbicide (GBH) during gestational and suckling periods, previously observed in immature offspring hippocampus, persist until adulthood in rats. Wistar rats were exposed to 1% GBH in drinking water (corresponding to 0.36% of glyphosate) from gestational day 5 until postnatal day (PND) 15 or PND60. The GBH source was Roundup Original (Brazil) containing glyphosate 360 g/L. The authors evaluated several mechanistic or neurochemical endpoints, as well as the behavioural alterations induced by the subchronic GBH exposure in adult animals (PND60). Also, the authors assessed if glyphosate might affect the glutamate neurotransmission system by using molecular docking of glyphosate to glycine and glutamate binding sites on the N-methyl-D-aspartate (NMDA) glutamate receptors.
Short description of findings	Subchronic GBH exposure during both prenatal and postnatal periods caused oxidative stress, and affected cholinergic and glutamatergic neurotransmission in offspring hippocampus from immature and adult rats. The subchronic GBH exposure decreased L-[^{14}C] glutamate uptake and increased $^{45}Ca^{2+}$ influx in 60-day-old rat hippocampus, suggesting persistent glutamate excitotoxicity from developmental period (PND15) to adulthood (PND60). Moreover, GBH exposure altered the serum levels of the astrocytic protein S100B. The effects of GBH exposure were associated with oxidative stress and depressive-like behaviour in offspring on PND60, as demonstrated by the prolonged immobility time and decreased time of climbing observed in a forced swimming test. The authors concluded that the underlying mechanisms for GBH-induced neurotoxicity involved the NMDA receptor activation, impairment of cholinergic transmission, astrocyte dysfunction, ERK1/2 overactivation, decreased p65 NF-κB phosphorylation, which are associated with oxidative stress and glutamate excitotoxicity. These neurochemical events might contribute to the depressive-like behaviour observed in adult offspring. The acetylcholinesterase inhibition also suggested the involvement of the cholinergic system, in addition to the glutamatergic one, in the neurotoxicity induced by GBH in rat hippocampus.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Roundup Original, Brazil, 360 g/L glyphosate) differs in overall composition to the EU representative formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Clair E. *et al.*
Year	2012
Title	A glyphosate based herbicide induces necrosis and apoptosis in mature rat testicular cells *in vitro*, and testosterone decrease at lower levels
Document source	Toxicology *In vitro* (2012), Vol. 26, No. 2, pp. 269-79
Short description of literature article	Roundup is being used increasingly in particular on genetically modified plants grown for food and feed that contain its residues. Glyphosate, its active ingredient in plants, and its metabolite (AMPA) are among the first contaminants of surface waters. This study aimed to measure the differential specificities of glyphosate and its formulation Roundup on adult rat freshly separated testicular cells to know the threshold of toxicity. The studied cell types were Leydig, Sertoli cells exposed in association or not with germ cells, and germ cells alone. The authors assayed necrosis and apoptosis at sub agricultural dilutions of glyphosate and Roundup, and tested the endocrine disruption at non cytotoxic levels from 1 to 10000 ppm, thus from the range in some human urine and the environment to agricultural levels. Glyphosate was purchased from Sigma Aldrich (France), and the used herbicide was a commercial formulation Roundup Bioforce containing 360 g/L of acid glyphosate. This work was the first study on the side effects of the main herbicide of the world on primary testicular mammalian cells.
Short description of findings	The authors showed that Leydig cells were damaged from 1 to 48 h of Roundup exposure from 0.1% (1000 ppm). Within 24 to 48 h, this formulation was also toxic on the other cells, mainly by necrosis. In contrast, glyphosate alone had no action at any time over 24 h in Leydig cells and exerted a very light action on caspases 3/7 activities after 48 h. Sertoli cells were almost insensitive to herbicide induced mortality, whereas glyphosate alone was essentially toxic on these cells. In 48 h, glyphosate from 0.5% (5000 ppm) also induced apoptosis at higher doses in germ cells and Sertoli/germ cells co-cultures. At lower non toxic concentrations of Roundup and glyphosate (1 ppm), the main endocrine disruption was a testosterone decrease by 35%. Roundup had thus an endocrine impact at very low environmental doses, but only high contamination appeared to provoke an acute rat testicular toxicity. This does not predict the chronic toxicity, which is insufficiently tested and only with glyphosate in regulatory tests. The authors considered that the presence of adjuvants in commercial formulations is decisive in inducing most herbicide side effects, postulating that the present regulatory *in vivo* tests with glyphosate alone to study chronic toxicity are hardly relevant.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the glyphosate EU renewal and risk assessment because a glyphosate formulation (Roundup Bioforce) was used for *in vitro* testing instead of glyphosate. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	de Liz Oliveira Cavalli V. L. *et al.*
Year	2013
Title	Roundup disrupts male reproductive functions by triggering calcium mediated cell death in rat testis and Sertoli cells.
Document source	Free Radical Biology & Medicine (2013), Vol. 65, pp. 335-46
Short description of literature article	This study aimed to assess acute exposure of immature rat testis to low doses of Roundup as a model of toxicity to the male reproductive system. Therefore, the authors investigated the molecular basis of the toxicity of this xenobiotic, focusing on the role of Ca^{2+} homeostasis, misregulation of signalling pathways, and oxidative damage in the whole rat testis or Sertoli cells in culture from 30-day-old Wistar male rats. This study used the commercial formulation Roundup Original (Brazil) containing glyphosate 360 g/L. Several analyses were performed, including $^{45}Ca^{2+}$ uptake, measurement of lactate dehydrogenase activity, amino acid accumulation experiments, antioxidant enzyme assays, γ-glutamyltransferase assay, glucose-6-phosphate dehydrogenase assay, catalase activity, superoxide dismutase activity, glutathione enzymes activity, endogenous lipid peroxidation, and protein carbonyl assay.
Short description of findings	The authors showed that acute Roundup exposure at low doses (36 ppm, 0.036 g/L) for 30 min induced oxidative stress and activated multiple stress response pathways leading to Sertoli cell death in prepubertal rat testis. Roundup increased intracellular Ca^{2+} concentration by opening L type voltage dependent Ca^{2+} channels as well as endoplasmic reticulum IP3 and ryanodine receptors, leading to Ca^{2+} overload within the cells, which set off oxidative stress and necrotic cell death. Similarly, 30 min incubation of testis with glyphosate alone (36 ppm) also increased $^{45}Ca^{2+}$ uptake. Activated protein kinase C, phosphatidylinositol-3-kinase, and the mitogen-activated protein kinases such as ERK1/2 and p38MAPK played a role in eliciting Ca^{2+} influx and cell death. Roundup decreased the levels of reduced glutathione (GSH) and increased the amounts of thiobarbituric acid-reactive species and protein carbonyls. Also, exposure to glyphosate Roundup stimulated the activity of glutathione peroxidase, glutathione reductase, glutathione S-transferase, γ-glutamyltransferase, catalase, superoxide dismutase, and glucose 6 phosphate dehydrogenase, supporting downregulated GSH levels. The authors proposed that Roundup toxicity, implicated in Ca^{2+} overload, cell signalling misregulation, stress response of the endoplasmic reticulum, and/or depleted antioxidant defences, could contribute to Sertoli cell disruption in spermatogenesis, which could have an impact on male fertility.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested *in vitro* was Roundup Original, 360 g/L a.e., Brazil. As this formulation differs in composition and co formulants to the representative formulation MON 52276, the article is not considered relevant to the EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	de Oliveira Joaquim A. *et al.*
Year	2014
Title	Effects of exposure to glyphosate in male and female mice behavior in pubertal period
Document source	Brazilian Journal of Veterinary Research and Animal Science (2014), Vol. 51, No. 3, pp. 194-203
Short description of literature article	This study aimed to investigate the effects of pre pubertal exposure of male and female mice to a commercial formulation of glyphosate on sexual dimorphism observed in animal models of emotionality, anxiety and depression. Therefore, adult BALB/c mice were exposed from 23 days of age (PND) until PND 45 to glyphosate (50 mg/kg, per os) or saline solution. Ten days after the end of treatments, male and female mice were observed in the open field (OF), elevated plus maze (EPM) or forced swimming test (FWT). The experiments were performed using Roundup Transorb (oral administration, 480 g/L of glyphosate, 648 g/L of isopropylamine salt and 594 g/L of inert ingredients; Monsanto, Brazil).
Short description of findings	This study showed that exposure to glyphosate reduced the locomotion frequency in the OF studies of male and female mice, compared to the corresponding control groups. Female mice had an increase in rearing behaviour and in the immobility time. Moreover, exposure to glyphosate reduced the motor activity in male mice both in the OF and EPM studies, while no effects were observed in female mice. Finally, in the FWT, the male mice had increased latency to float and decreased time of float, while the female mice presented decreased latency to float and increased time of float. The authors concluded that pre pubertal exposure to glyphosate reduced the capacity of exploration in male mice in the OF and EPM studies, suggesting that the herbicide interfered with the central mechanism related to brain masculinization of exploratory and anxiety behavioural models.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate because a formulation (Roundup Transorb) was used instead of glyphosate. The data on this formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study differs in composition to the EU representative formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	de Souza J. S. *et al*.
Year	2017
Title	Perinatal exposure to glyphosate based herbicide alters the thyrotrophic axis and causes thyroid hormone homeostasis imbalance in male rats
Document source	Toxicology (2017), Vol. 377, pp. 25-37
Short description of literature article	This study aimed to verify the potential impact of a commercial GBH on the hypothalamic-pituitary-thyroid (HPT) axis to establish the risk for endocrine system disruption. Female pregnant Wistar rats were exposed to a solution containing GBH Roundup Transorb (Monsanto, Brazil) diluted in water. The animals were divided into three groups (control, 5 mg/kg/day or 50 mg/kg/day) and exposed from gestation day 18 (GD18) to postnatal day 5 (PND5). Male offspring were euthanized at PND 90, and blood and tissues samples from the hypothalamus, pituitary, liver and heart were collected for hormonal evaluation (TSH-Thyroid stimulating hormone, T3-triiodothyronine and T4-thyroxine), metabolomic and mRNA analyses of genes related to thyroid hormone metabolism and function.
Short description of findings	The hormonal profiles showed decreased concentrations of TSH in the exposed groups, with no variation in the levels of the thyroid hormones (THs) T3 and T4 between the groups. Hypothalamus gene expression analysis of the exposed groups revealed a reduction in the expression of genes encoding deiodinases 2 (Dio2) and 3 (Dio3) and TH transporters Slco1c1 (former Oatp1c1) and Slc16a2 (former Mct8). In the pituitary, Dio2, thyroid hormone receptor genes (Thra1 and Thrb1), and Slc16a2 showed higher expression levels in the exposed groups than in the control group. In contrast, Tshb gene expression did not show any difference in expression profile between the control and exposed groups. Liver Thra1 and Thrb1 showed increased mRNA expression in both GBH-exposed groups, and in the heart, Dio2, Mb, Myh6 (former Mhca) and Slc2a4 (former Glut4) showed higher mRNA expression in the exposed groups. Additionally, correlation analysis between gene expression and metabolomic data showed similar alterations as detected in hypothyroid rats. Perinatal exposure to GBH in male rats modified the HPT set point, with lower levels of TSH likely reflecting post translational events. Several genes regulated by TH or involved in TH metabolism and transport presented varying degrees of gene expression alteration that were probably programmed during intrauterine exposure to GBHs and reflects in peripheral metabolism. The authors concluded that changes in the programming of the HPT axis might occur after GBH exposure, and more studies, particularly epidemiological studies, are needed to clarify the precise effect of this ED on the HPT axis and develop strategies for public health actions.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the glyphosate EU renewal and risk assessment because a glyphosate formulation (Roundup Transorb) was used instead of glyphosate. The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study differs in composition and co-formulants to the EU representative formulation MON 52276. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	de Souza J. S. *et al.*
Year	2019
Title	Maternal glyphosate based herbicide exposure alters antioxidant related genes in the brain and serum metabolites of male rat offspring
Document source	Neurotoxicology (2019), Vol. 74, pp. 121-131
Short description of literature article	Although several epidemiological studies have demonstrated that there are health risks associated with glyphosate based herbicides (GBHs) exposure, the effects these chemicals have on the oxidative and inflammatory response in the brain are still unclear. Alterations in these processes could contribute to the development of neurological diseases, such as Alzheimer's disease and autism spectrum disorders. This report study exposed pregnant rats to GBH and evaluated changes in the expression of genes related to oxidative stress and inflammation response and monitored the serum metabolome in the cortex and cerebellum of adult male offspring. Serum metabolomics was performed to determine if the changes in metabolite concentrations could be correlated with modifications in gene expression. Pregnant Wistar rats were administered distilled water or Roundup Transorb (Monsanto, Brazil), at either 5 and 50 mg/kg/day from gestational day (GD) 18 to postnatal day (PND) 5.
Short description of findings	There was a significant increase in the gene expression levels of Neuroglobin (Ngb, oxygen storage and tissue protection) (105%, p = 0.031), Glutathione Peroxidase 1 (Gpx1, oxidative stress) (95%, p = 0.005), Prostaglandin Endoperoxidase Synthase 1 (Ptgs1, inflammation) (109%, p = 0.033) and Hypoxia inducible factor 1 subunit alpha (Hif1α, oxygen sensor) (73%, p = 0.017), in the cerebellum of PND90 rats perinatally exposed to 50 mg GBH/kg/day. Moreover, both GBH exposed groups displayed a significant decrease in the expression of Catalase (Cat, oxidative stress) (49%, p = 0.003; and 31% p = 0.050, respectively) expression, in the cortex. Serum metabolites analyses, from the same animals of each group, demonstrated that there were significant changes in the concentrations of lysophosphatidylcholine and phosphatidylcholine, which have been associated with neurodegenerative diseases. The authors suggested that GBH exposure during pregnancy alters the expression of genes associated with oxidant defence, inflammation and lipid metabolism. It is plausible that maternal GBH exposure could have lasting neuronal effects on the offspring later in life.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulated product tested was Glyphosate Roundup Transorb (Monsanto of Brazil Ltda, São Paulo, Brazil). As this is not the EU representative formulation, the article is not considered relevant to the EU renewal of glyphosate. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. In addition, the test item administration does not represent an operator or consumer exposure route of potential exposure to glyphosate.

Data point	CA 9
Author	Emmanuel A. G. *et al.*
Year	2015
Title	Protective potential of betulinic acid against glyphosate induced toxicity in testis and epididymis of male wistar rats
Document source	International Journal of Current Research (2015), Vol. 7, No. 6, pp. 16650-16660
Short description of literature article	This study examined possible protective potentials of betulinic acid (BA) on antioxidant parameters and histology of testis and epididymis of glyphosate treated rats. Twenty male Wistar rats were randomly divided into four groups: control, administered with BA (10 mg/kg) every other day, treated with glyphosate (100 mg/kg) on days 4, 6, 10, and 12, or pretreated with BA (10 mg/kg) on days 1 and 3 and glyphosate (100 mg/kg) on days 4, 6, 10, and 12. The authors reported using commercial glyphosate for the experiments. Preparations of serum and tissue homogenates were analysed for the determination of total protein, lipid peroxidation, reduced glutathione (GSH), superoxide dismutase (SOD) activity, catalase (CAT) activity, and histopathological characteristics.
Short description of findings	Glyphosate significantly elevated malondialdehyde (MDA) in testes and epididymis by 41.8% and 49.5%, respectively, compared with controls. BA significantly decreased MDA by 47.8% and 34.0%, respectively, compared with glyphosate group. SOD was significantly reduced by 66.7% and 73.7% in testes and epididymis, in glyphosate group, whereas BA supplementation significantly elevated ($p < 0.05$) SOD by 77.8% and 72.2% respectively. CAT activities were reduced in the glyphosate group by 29.2% and 21.7% in testes and epididymis, while BA elevated CAT activities by 23.8% and 28.0% respectively. Glyphosate decreased the level of GSH by 35.2% in testes, compared with controls, while BA supplementation significantly increased ($p < 0.05$) GSH level by 43.8%, compared with glyphosate group. Glyphosate induced cellular degeneration and deformity in testis and epididymis. The authors concluded that glyphosate disrupted the antioxidant defence system and histological features of testicular and epididymal tissues, whereas betulinic acid exhibited the potential to reverse these toxic effects of the herbicide in male rats.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested *in vivo* was described as "commercial glyphosate". The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Gallegos C. E. *et al.*
Year	2016
Title	Exposure to a glyphosate based herbicide during pregnancy and lactation induces neurobehavioral alterations in rat offspring
Document source	NeuroToxicology (2016), Vol. 53, pp. 20-28
Short description of literature article	The purpose of the present study was to assess the neurobehavioral effects of chronic exposure to a glyphosate containing herbicide during pregnancy and lactation. The pesticide used in this study is a commercial formulation marketed in Argentina as Glifloglex from Gleba S.R.L., which contains 48 g of glyphosate isopropylamine salt per 100 cm^3 product (equivalent to 35.6% w/v of glyphosate acid). Pregnant Wistar rats were supplied orally with 0.2% or 0.4% of this glyphosate formulation corresponding to a concentration of 0.65 or 1.30 g/L of glyphosate, respectively. During the complete gestational and lactation periods, and offspring were subjected to a series of neurobehavioral tests. The postnatal day (PND) on which each pup acquired neonatal reflexes (righting, cliff aversion and negative geotaxis) and that on which eyes and auditory canals were fully opened were recorded for the assessment of sensorimotor development. Furthermore, locomotor activity and anxiety levels were analysed in 45 and 90 day old offspring by means of the open field test and plus maze test, respectively.
Short description of findings	Pups exposed to a glyphosate based herbicide showed early onset of cliff aversion reflex and early auditory canal opening. A decrease in locomotor activity and in anxiety levels was also observed in the groups exposed to a glyphosate-containing herbicide. Findings from the present study reveal that early exposure to a glyphosate-based herbicide affects the central nervous system in rat offspring probably by altering mechanisms or neurotransmitter systems that regulate locomotor activity and anxiety.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested *in vivo* via drinking water (Glifloglex, 48% glyphosate, Gleba S.R.L., Argentina). As this is not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Gallegos C. E. *et al.*
Year	2018
Title	Perinatal glyphosate based herbicide exposure in rats alters brain antioxidant status, glutamate and acetylcholine metabolism and affects recognition memory
Document source	Neurotoxicity Research (2018), Vol. 34, No. 3, pp. 363-374
Short description of literature article	The aim of this work was to elucidate the possible mechanisms underlying the neurotoxicity exerted by chronic exposure to a commercial glyphosate containing herbicide during pregnancy and lactation. The pesticide used in this study is a commercial formulation marketed in Argentina as Glifloglex from Gleba S.R.L., which contains 48 g of glyphosate isopropylamine salt per 100 cm^3 product (equivalent to 35.6% w/v of glyphosate acid), together with an unspecified mix of inerts and adjuvants. Pregnant Wistar rats were supplied orally with this glyphosate formulation during the complete gestational and lactation periods, and biochemical and neuroconductual tests were performed on 90 days pups. Oxidative stress markers were determined in the whole brain, and the activities of the enzymes AChE, transaminases, and alkaline phosphatase were assessed in specific brain areas (prefrontal cortex, striatum, and hippocampus), which are related with the neuroconductual disorders observed in previous studies. In addition, the offspring were subjected to novel object recognition test in order to analyse if glyphosate based herbicide exposure affects recognition memory. Sexually mature male and female Wistar rats (90–120 days old) from our own breeding center were used. They were maintained under constant temperature (22 ± 1 °C) and humidity (50–60%) conditions in a 12-h light-dark-cycle, with food (Ganave, Alimentos Pilar S.A., Argentina) and water *ad libitum*.
Short description of findings	Brain antioxidant status was altered in glyphosate based herbicide exposed rats. Moreover, AChE and transaminases activities were decreased and AP activity was increased in PFC, striatum and hippocampus by glyphosate-based herbicide treatment. In addition, the recognition memory after 24 h was impaired in adult offspring perinatally exposed to glyphosate-based herbicide. The present study reveals that exposure to a glyphosate-based herbicide during early stages of rat development affects brain oxidative stress markers as well as the activity of enzymes involved in the glutamatergic and cholinergic systems. These alterations could contribute to the neurobehavioral variations reported previously, and to the impairment in recognition memory described in the present work.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The formulation tested (Glifloglex) is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	George J. *et al.*
Year	2010
Title	Studies on glyphosate induced carcinogenicity in mouse skin: a proteomic approach.
Document source	Journal of Proteomics (2010), Vol. 73, No. 5, pp. 951-64
Short description of literature article	The present investigation was carried out to study the carcinogenic potential of glyphosate and to identify differentially expressed proteins, using 2-dimensional gel electrophoresis and mass spectrometry analysis after treatment with glyphosate, a known tumour promoter, 12-o-tetradecanoyl-phorbol-13-acetate (TPA) and tumour initiator, 7, 12-dimethylbenz[a]anthracene (DMBA) in mouse skin. As test item commercial formulation of the herbicide glyphosate Roundup Original (glyphosate 41%, POEA ≅15%, Monsanto Company, St. Louis, MO, USA) was used, which contains 360 g/L glyphosate acid equivalent as the isopropylamine salt and was procured from a local market. Male Swiss albino mice (12-15 g body weight [bw]) were taken from the Indian Institute of Toxicology Research (IITR) animal breeding colony and acclimatized for 1 week. The ethical approval for the experiment was obtained from the institutional ethical committee. The animals were kept under standard laboratory conditions (temperature 23 ± 2 °C, relative humidity 55 ± 5%) and were fed with synthetic pellet basal diet (Ashirwad, Chandigarh, India) and tap water *ad libitum*. Animals were randomly divided into 8 groups of 20 animals each.
Short description of findings	Proteomic analysis using 2-dimensional gel electrophoresis and mass spectrometry showed that 22 spots were differentially expressed (>2 fold) on glyphosate, DMBA and TPA application over untreated control. Among them, 9 proteins (translation elongation factor eEF-1 alpha chain, carbonic anhydrase III, annexin II, calcyclin, fab fragment anti-VEGF antibody, peroxiredoxin-2, superoxide dismutase [Cu–Zn], stefin A3, and calgranulin-B) were common and showed similar expression pattern in glyphosate and TPA-treated mouse skin. These proteins are known to be involved in several key processes like apoptosis and growth-inhibition, anti-oxidant responses, etc. The upregulation of calcyclin, calgranulin-B and downregulation of superoxide dismutase [Cu–Zn] was further confirmed by immunoblotting, indicating that these proteins can be good candidate biomarkers for skin carcinogenesis induced by glyphosate. Altogether, these results suggested that glyphosate has tumour promoting potential in skin carcinogenesis and its mechanism seems to be similar to TPA.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The test material was Roundup Original (containing POEA) and not the reference formulation MON 52276. As this is not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Gomez A. L. *et al.*
Year	2019
Title	Male mammary gland development and methylation status of estrogen receptor alpha in Wistar rats are modified by the developmental exposure to a glyphosate based herbicide
Document source	Molecular and Cellular Endocrinology (2019), Vol. 481, pp. 14-25
Short description of literature article	Aims of the present study were to determine whether oral developmental exposure to a glyphosate based herbicide (GBH) affects mammary gland growth and development in pre- and post-pubertal male rats, and to evaluate the possible mechanisms involved. For this, pregnant rats (F0) on gestational day 9 (GD9) were weighed and randomly divided into three groups (8–10 dams/group): a) Control, fed with a standard diet (laboratory pellet chow, 16–014007 Rat Mouse diet, Nutrición Animal, Santa Fe, Argentina); b) GBH3.5, fed with a standard diet supplemented with a GBH (Magnum Super II, Grupo Agros SA; Glyphosate 66.2% - potassium salt, acid equivalent 54%) in a dose of 3.7 mg of glyphosate/kg bw/day; and c) GBH350, fed with a standard diet supplemented with a GBH (Magnum Super II) in a dose of 352 mg of glyphosate/kg bw/day. The doses of glyphosate were selected based on the no-observed-adverse-effect level (NOAEL) of 1000 mg/kg bw/day for maternal and developmental toxicity established for this herbicide by the US EPA and were in the order of magnitude and a 100-times lower than the NOAEL of 300 mg/kg bw/day specified by the EFSA. Mammary gland development and estradiol (E2) and testosterone (T) serum levels of male offspring were evaluated on postnatal day (PND) 21 and PND60. Besides, prolactin (PRL) serum levels, proliferation index, androgen (AR) and estrogen receptor alpha (ESR1) expression, ESR1 alternative transcript mRNA levels, and DNA methylation status of ESR1 promoters were assessed on PND60.
Short description of findings	No differences between groups were observed in mammary gland development at PND21 or in E2 and T levels on both PNDs studied. On PND60, GBH3.5-exposed animals presented similar mammary gland histology but higher AR protein expression than controls, whereas GBH350-exposed males presented a less developed mammary gland, accompanied by a lower proliferation index, similar AR levels, and slightly increased PRL serum levels than controls. In both exposed groups, ESR1 expression was lower than in control rats, being lower in GBH350-exposed rats. GBH also altered the abundance of ESR1 transcript variants by hypermethylation of ESR1 promoters. GHB3.5 decreased only ESR1-OS expression, whereas GBH350 affected ESR1-O, OT and E1 expression. Our results show that developmental exposure to GBH induces epigenetic changes in ESR1, which could be responsible for the altered male mammary gland development observed in GBH350-exposed animals.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Magnum Super II, Grupo Agros SA; 66.2% potassium salt, 54% a.e.) is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Guerrero Schimpf M. *et al.*
Year	2017
Title	Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus
Document source	Toxicology (2017), Vol. 376, pp. 2-14
Short description of literature article	This study evaluated if neonatal exposure to a glyphosate based herbicide (GBH) affects uterine morphology, proliferation and expression of proteins that regulate uterine organogenetic differentiation in rats. Female Wistar pups received saline solution (control, C) or a commercial formulation of glyphosate (GBH, 2 mg/kg) by subcutaneous injection every 48 h from postnatal day (PND) 1 to PND7. Rats were sacrificed on PND8 (neonatal period) and PND21 (prepubertal period) to evaluate acute and short term effects, respectively. The uterine morphology was evaluated in haematoxylin and eosin stained sections. The epithelial and stromal immunophenotypes were established by assessing the expression of luminal epithelial protein (cytokeratin 8; CK8), basal epithelial proteins (p63 and pan cytokeratin CK1, 5, 10 and 14); and vimentin by immunohistochemistry (IHC). To investigate changes on proteins that regulate uterine organogenetic differentiation the study evaluated the expression of oestrogen receptor alpha (ERα), progesterone receptor (PR), Hoxa10 and Wnt7a by IHC.
Short description of findings	GBH-exposed uteri showed morphological changes, characterized by an increase in the incidence of luminal epithelial hyperplasia (LEH) and an increase in the stromal and myometrial thickness. The epithelial cells showed a positive immunostaining for CK8, while the stromal cells for vimentin. GBH treatment increased cell proliferation in the luminal and stromal compartment on PND8, without changes on PND21. GBH treatment also altered the expression of proteins involved in uterine organogenetic differentiation. PR and Hoxa10 were deregulated both immediately and two weeks after the exposure. ERα was induced in the stromal compartment on PND8, and was downregulated in the luminal epithelial cells of glyphosate exposed animals on PND21. GBH treatment also increased the expression of Wnt7a in the stromal and glandular epithelial cells on PND21. Neonatal exposure to GBH disrupts the postnatal uterine development at the neonatal and prepubertal period. All these changes may alter the functional differentiation of the uterus, affecting female fertility and/or promoting the development of neoplasias.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested *in vivo* via subcutaneous injection (Roundup FULL II, 66.2% potassium salt). As this is not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Guerrero Schimpf M. K
Year	2018
Title	Glyphosate based herbicide enhances the uterine sensitivity to estradiol in rats
Document source	The Journal of Endocrinology (2018), Vol. 239, No. 2, pp. 197-213
Short description of literature article	The goal of the study was to determine whether exposure to a low dose of a GBH during postnatal development might enhance the sensitivity of the uterus to an estrogenic treatment. All procedures used in this study were performed in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals issued by the US National Academy of Sciences and were approved by the Institutional Ethics Committee of the School of Biochemistry and Biological Sciences (Universidad Nacional del Litoral, Santa Fe, Argentina). Inbred Wistar strain rats were bred at the Department of Human Physiology (Santa Fe, Argentina) and housed in a controlled environment (22°C ± 2°C; lights on from 06:00 to 20:00 h) in stainless steel cages with sterile pine wood shavings as bedding. Rats had free access to pellet laboratory chow (16-014007 Rat Mouse Diet, Nutrición Animal, Santa Fe, Argentina) and tap water. Female Wistar pups were subcutaneously injected with saline solution (control) or GBH using the reference dose (2 mg/kg/day, EPA) on postnatal days (PND) 1, 3, 5 and 7. At weaning (PND21), female rats were bilaterally ovariectomized and treated with silastic capsules containing 17β-estradiol (E2, 1 mg/mL) until they were 2 months of age. On PND60, uterine samples were removed and processed for histology, immunohistochemistry and mRNA extraction to evaluate: (i) uterine morphology, (ii) uterine cell proliferation by the detection of Ki67, (iii) the expression of the oestrogen receptors alpha (ESR1) and beta (ESR2) and (iv) the expression of WNT7A and CTNNB1.
Short description of findings	GBH-exposed animals showed increased luminal epithelial height and stromal nuclei density. The luminal and glandular epithelium were markedly hyperplastic in 43% of GBH-exposed animals. GBH exposure caused an increase in E2-induced cell proliferation in association with an induction of both ESR1 and ESR2. GBH treatment decreased membranous and cytoplasmic expression of CTNNB1 in luminal and glandular epithelial cells and increased WNT7A expression in the luminal epithelium. These results suggest that early postnatal exposure to a GBH enhances the sensitivity of the rat uterus to estradiol and induces histomorphological and molecular changes associated with uterine hyperplasia.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Glyphosate formulation used was Roundup FULL II, a liquid water-soluble formulation containing 66.2% of glyphosate potassium salt. As this is not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Hamdaoui L. *et al.*
Year	2018
Title	Subchronic exposure to kalach 360 SL-induced endocrine disruption and ovary damage in female rats
Document source	Archives of Physiology and Biochemistry (2018), Vol. 124, No. 1, pp. 27-34
Short description of literature article	The aim of this study is to evaluate subchronic exposure of two different doses of Kalach 360 SL, a glyphosate-based herbicide, a low dose and a high dose, on rats' female reproductive system. Specifically, the study investigated the morphological and biochemical aspects of ovary injury after exposure to KL. For these thirty mature female rats, weighing about 200-220g, were purchased from the Central Pharmacy (SIPHAT, Tunisia). The rats were kept under controlled conditions of temperature (22°C), humidity (60%) and 12/12h light dark cycle with 07:30 a m.–7:30 p.m. being the light phase. The herbicide used in this study was the commercial glyphosate formulation Kalach 360 SL (KL) containing the active ingredient as isopropylamine salt of n-phosphonomethylglycine (41.5%), surfactant (15.5%) and water (43%). Female adults Wistar rats were free to access the commercial pellet diet (SICO, Sfax, Tunisia) and water *ad libitum* and they were divided into three groups: Group 1 which served as control received a standard diet (n = 6), Group 2 was composed of 12 rats. Each one received by gavage 0.07 mL of KL dissolved in 1 mL of water. This dose contained 126 mg of glyphosate/kg (dose 1), Group 3 was composed of 12 rats. Each one received by gavage 0.175 mL of KL, dissolved in 1 mL of water. This dose contained 315 mg of glyphosate/kg (dose 2).
Short description of findings	The subchronic exposure of KL induces impaired folliculogenesis, ovary development, decreased oestrogen secretion, promoted oxidative stress and impairments of ovary histological aspects. Histological findings show necrosis cell, vacuolisation of follicles, dissociated oocytes and granulosa cell, associated with several atretic follicles. The researchers conclude that KL induces endocrine disruption and ovary damage in female rats.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (Kalach 360 SL from Arysta Life Science, Tunisia) is not the EU representative formulation, therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Hao Y. *et al.*
Year	2019
Title	Roundup confers cytotoxicity through DNA damage and mitochondria-associated apoptosis induction
Document source	Environmental Pollution (2019), Vol. 252, No. Part A, pp. 917-923
Short description of literature article	In this study, human alveolar carcinoma A549 cells (served as models of alveolar Type II pulmonary epithelium) were selected to detect the Roundup toxicity on lung tissue and its potential risk of inhalation toxicity to humans (e.g. mitochondria associated apoptosis and DNA damage). Alkaline comet assay, immunofluorescence assay and flow cytometric analysis assay were employed as test systems. Roundup (RDP) was obtained from Monsanto (St. Louis, Missouri, USA). Test formulations RDP (0, 50, 75, 100 and 125 mg/mL, calculated based on the active ingredient glyphosate) were freshly prepared in double-distilled water. Trypan Blue, Rhodamine123, phenylmethanesulfonyl fluoride, 4',6-diamidino-2-phenylindole, propidium iodide (PI), and radioimmunoprecipitation assay lysis buffer were obtained from Sigma (St. Louis, MO, USA). All other chemicals and reagents used were obtained from Shanghai Titanchem Co. Ltd. (Shanghai, China). Antibodies were used as follows: caspase-9 (35, 37 and 47 kDa), Bax (20 kDa), Bcl-2 (26 kDa), caspase-3 (35 kDa), cytochrome c (14 kDa), PARP (89 and 116 kDa), gH2AX (14 kDa), and β actin (43 kDa) from Cell Signaling Technology (Beverly, MA, USA). Secondary antibody was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Human alveolar carcinoma A549 cell line (ATCC, CCL-185) was maintained in DMEM (Hyclone, USA) supplemented with 1% antibiotics (streptomycin and penicillin) (Gibco, USA) and 10% fetal bovine serum (Gibco, USA), and maintained in 37 ºC, 5% CO2 in a humidified atmosphere.
Short description of findings	According to the authors, RDP induced the DNA single-strand breaks and double-strand breaks; the collapse of mitochondrial membrane by increasing Bax/Bcl-2, resulting in the release of cytochrome c into cytosol and then activated caspase-9/-3, cleaved poly (ADP-ribose) polymerase (PARP) in human lung tissue cells. The results demonstrate that RDP can induce A549 cells cytotoxic effects *in vitro* at the concentration lower than the occupational exposures level of workers, which means RDP has a potential threat to human health. However, based on the current research data presented in the study, the authors stated that they could not certain whether the cytotoxic effects of RDP are due to herbicidal active ingredient, adjuvants, or a combination of the two.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication was considered not relevant for the risk assessment and the glyphosate EU renewal because a glyphosate formulation was tested *in vitro* instead of glyphosate. Surfactants present in the formulation induce membrane damage and cytotoxicity, these effects are well established within *in vitro* test systems, and this research is not relevant or informative for the glyphosate EU renewal. In addition, the surfactant system in the formulation tested differs from the EU representative glyphosate formulation MON 52276. Therefore, the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Ikpeme E. V. *et al*.
Year	2012
Title	Efficacy of ascorbic acid in reducing glyphosate induced toxicity in rats
Document source	British Biotechnology Journal (2012), Vol. 2, No. 3, pp. 157-168
Short description of literature article	This study was aimed at investigating the effects of glyphosate on the sperm dynamics of male albino rats and the protective effects of ascorbic acid. For this purpose, twenty-five sexually matured male albino rats were purchased from the animal house of the Department of Zoology and Environmental Biology, University of Calabar, Calabar. Glyphosate was purchased from Cross River Agricultural Development Project (CRADP). The rats were maintained under standard conditions of humidity, temperature and standard chow (feed) and tap water was given *ad libitum*. The twenty-five rats were divided into five groups of five rats each in a completely randomised design (CRD). Rats in group 1 served as the control while rats in groups 2 and 3 received 250 mL/kg of glyphosate in the morning while rats in group 3 received 200 mg/kg of ascorbic acid dissolved in 1 mL of distilled water in the evening. Additionally, rats in group 4 and 5 received 500 mL/kg of glyphosate and group 5 rats received 200 mg/kg of ascorbic acid in the morning and evening. These administrations were done through oral gavage for 65 days (Note: the oral lethal dose of glyphosate for rat is 5000 mg/kg while ascorbic acid is 1900 mg/kg). At the end of the treatment regimen, they were sacrificed under chloroform anaesthesia. The testes and epididymes were dissected and weighed. Data were collected on the following parameters: sperm motility, sperm count, sperm viability, sperm morphology, semen pH and weight of organs (testes and epididymes).
Short description of findings	According to the authors, the study showed that there were significant adverse effects ($P < 0.05$) of glyphosate treatment on sperm parameters and the cytoarchitecture of the gonad, which showed disruption in the seminiferous tubules, necrotic germinal epithelium and clumped Leydig cells. However, administering the rats with ascorbic acid caused significant ameliorating effects on the parameters investigated.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Ingaramo P. I. *et al*.
Year	2016
Title	Effects of neonatal exposure to a glyphosate based herbicide on female rat reproduction
Document source	Reproduction (2016), Vol. 152, No. 5, pp. 403-15
Short description of literature article	The study aimed to investigate if postnatal exposure to a commercial glyphosate based herbicide (GBH) alters reproductive parameters in adult female rats and promotes failure of the endocrine regulated decidualization process. Effects of neonatal GBH exposure on (1) the reproductive performance by determining the pregnancy rate, the number of corpora lutea (CLs), and the number of implantation sites (IS) and resorption sites (RS) on GD19; and (2) the ovarian steroid levels, uterine morphology, endometrial cell proliferation, apoptosis and cell cycle regulators (p27 and cyclin G1), and the endocrine pathways that regulate uterine decidualization (steroid receptors/COUP TFII/Bmp2/Hoxa10) at the IS on GD9 were investigated. For this, newborn female rats received vehicle or 2 mg/kg/day of a GBH on postnatal days (PND) 1, 3, 5 and 7. On PND90, the rats were mated to evaluate (i) the reproductive performance on gestational day (GD) 19 and (ii) the ovarian steroid levels, uterine morphology, endometrial cell proliferation, apoptosis and cell cycle regulators, and endocrine pathways that regulate uterine decidualization (steroid receptors/COUP TFII/Bmp2/Hoxa10) at the IS on GD9.
Short description of findings	The GBH-exposed group showed a significant increase in the number of resorption sites on GD19, associated with an altered decidualization response. In fact, on GD9, the GBH-treated rats showed morphological changes at the IS, associated with a decreased expression of oestrogen and progesterone receptors, downregulation of COUP TFII (Nr2f2) and Bmp2 mRNA and increased expression of HOXA10 and the proliferation marker Ki67 (Mki67) at the IS. Authors concluded that alterations in endometrial decidualization might be the mechanism of GBH-induced post implantation embryo loss.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: A glyphosate based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Ingaramo P. I. *et al*.
Year	2017
Title	Neonatal exposure to a glyphosate based herbicide alters uterine decidualization in rats
Document source	Reproductive Toxicology (2017), Vol. 73, pp. 87-95
Short description of literature article	The hypothesis of the study was that early postnatal exposure to glyphosate based glyphosate (GBH) alters the uterine development and function by deregulation of the Wnt5a, Wnt7a and β-catenin signalling pathway. To test this hypothesis, the authors investigated the effects of a brief neonatal exposure to a low dose of GBH in the uteri of female rats at the following three time points: i) immediately after the end of the exposure period)postnatal day (PND) 8, neonatal period) to evaluate the acute response to GBH; ii) two weeks after the end of the exposure period (PND21, prepubertal period) to investigate the short term response; and iii) during pregnancy on GD9 to evaluate long term effects on uterine decidualization. In addition, the postnatal ontogenetic pattern and cellular distribution of uterine Wnt5a were evaluated in unexposed rats (between PND1 and PND35) and unexposed pregnant rats on GD3, 4 and 5. Female pups received vehicle or 2 mg/kg of GBH from postnatal day (PND) 1 to PND7. On PND8 and PND21, Wnt5a and β-catenin expression was evaluated in uterine samples. On gestational day (GD) 9, Wnt5a, Wnt7a and β-catenin expression and Dkk1 and sFRP4 mRNA were evaluated on implantation sites.
Short description of findings	On PND8, GBH-exposed rats showed increased Wnt5a and β-catenin expression in luminal epithelium (LE), whereas, on PND21, they showed increased Wnt5a and β-catenin expression in subepithelial stroma but decreased β-catenin expression in glandular epithelium. On GD9, GBH-exposed rats showed decreased Wnt5a and Wnt7a expression in the antimesometrial zone and LE respectively, without changes in β-catenin expression, while Dkk1 and sFRP4 were up- and down-regulated respectively. Taken together, the results of the present study evidence a deregulation of the Wnt pathways that regulate uterine decidualization when rats are neonatally treated with a low dose of GBH. In a critical period of gestation like the decidualization process, an imbalance of regulatory molecules as Wnt/β-catenin could cause gestation failure.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: A glyphosate based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Ingaramo P. I. *et al*.
Year	2019
Title	Acute uterine effects and long term reproductive alterations in postnatally exposed female rats to a mixture of commercial formulations of endosulfan and glyphosate
Document source	Food and Chemical Toxicology (2019), Vol. 134, pp. 110832
Short description of literature article	The present study evaluated the acute and long term effects of a brief postnatal exposure to endosulfan insecticide (EF), glyphosate-based herbicide (GBH; 66.2% glyphosate potassium salt containing coadjuvants and co-formulants), and a mixture of them. Route of administration to adults is not adequately described, but the vehicle is consistent with injection administration. The authors assessed: 1) the acute effects evaluating uterine differentiation on PND8, and 2) the long term effects determining the reproductive performance of female adult rats. After birth, female pups of Wistar rats received subcutaneous injections of saline solution (CONTROL), EF (600 µg/kg of bw/day), GBH (2mg/kg of bw/day) or a mixture (at the same doses) from postnatal day (PND) 1 to PND7. The uterine histology and expression of Hoxa10, oestrogen (ERα) and progesterone (PR) receptors were evaluated on PND8. Reproductive performance was evaluated on gestational day 19.
Short description of findings	Rats exposed to the GBH formulation and or the mixture of GBH and endosulfan showed an increment of 1) the incidence of luminal epithelial hyperplasia, 2) PR and Hoxa10 expression. EF modified ERα and Hoxa10 expression. During adulthood, MIX and GBH rats showed higher post-implantation losses while EF alone produced an increase of preimplantation losses. The researchers showed that the co-administration of both pesticides produced acute uterine effects and long-term deleterious reproductive effects that were similar to those induced by GBH alone.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: A glyphosate-based herbicide formulation (66.2%, potassium salt) was tested *in vivo* via subcutaneous injection. As this is not a relevant route of exposure and not the EU representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Ji Hua *et al*.
Year	2017
Title	Differential microRNA expression in the prefrontal cortex of mouse offspring induced by glyphosate exposure during pregnancy and lactation
Document source	Experimental and Therapeutic Medicine (2018), Vol. 15, No. 3, pp. 2457-2467
Short description of literature article	In the present study, miRNA expression patterns were evaluated in the prefrontal cortex (PFC) of 28 postnatal day mouse offspring following glyphosate exposure during pregnancy and lactation. An miRNA microarray detected 55 upregulated and 19 downregulated miRNAs in the PFC of mouse offspring, and 20 selected deregulated miRNAs were further evaluated by quantitative polymerase chain reaction (PCR). A total of 11 targets of these selected deregulated miRNAs were analysed using bioinformatics. Gene Ontology (GO) terms associated with the relevant miRNAs included neurogenesis (GO:0050769), neuron differentiation (GO:0030182) and brain development (GO:0007420). The genes Cdkn1a, Numbl, Notch1, Fosl1 and Lef1 are involved in the Wnt and Notch signalling pathways, which are closely associated with neural development. PCR arrays for the mouse Wnt and Notch signalling pathways were used to validate the effects of glyphosate on the expression pattern of genes involved in the Wnt and Notch pathways.
Short description of findings	Nr4a2 and Wnt7b were downregulated, while Dkk1, Dixdc1, Runx1, Shh, Lef-1 and Axin2 were upregulated in the PFC of mice offspring following glyphosate exposure during pregnancy and lactation. These results indicated abnormalities of the Wnt/β-catenin and Notch pathways. These findings may be of particular interest in understanding the mechanism of glyphosate-induced neurotoxicity, as well as helping to clarify the association between glyphosate and NDDs. Baseline historical control data for these unique endpoints were not provided in order to understand the context of the results.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Glyphosate-based formulation (purchased in China, containing 48% IPA salt) was administered via gavage to mice with the endpoints measured not suitable for risk assessment (differential microRNA expression in the prefrontal cortex). The exact composition of the formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Jiang X. *et al*.
Year	2018
Title	A commercial Roundup formulation induced male germ cell apoptosis by promoting the expression of XAF1 in adult mice
Document source	Toxicology Letters (2018), Vol. 296, pp. 163-172
Short description of literature article	The aim of the present study, carried out in mice testis and a mouse spermatocyte cell line (GC-2), was to examine the role of apoptosis in the male reproductive toxicity of Roundup and intracellular mechanisms involved in the apoptotic event (e.g. the role of a novel tumour suppressor XAF1 (X-linked inhibitor of apoptosis-associated factor 1). For this adult 8-week-old male Kunming mice approximately 34 g (n = 32) were obtained from the Experimental Animal Center of the Army Medical University. The animals were maintained under pathogen free conditions with a 12-h light/12-h dark schedule and were fed with an autoclaved diet and water *ad libitum*. Thirty-two mice were randomly allocated into 4 groups (n = 8 per group): CG (control group), LDG (low dose group, containing 60 mg/kg of glyphosate), MDG (middle-dose group, containing 180 mg/kg glyphosate), and HDG (high-dose group, containing 540 mg/kg glyphosate). Animals were administered the indicated doses of Roundup diluted in distilled water or with distilled water alone as a control each day by gavage. For the glyphosate treatments Roundup formulation (Monsanto Co, St. Louis, MO, USA) contained 360 g/L of glyphosate and 18% (w/v) POEA (surfactant) were used. Sperm motility and concentration analysis by the sperm class analyser, epididymal sperm morphology analysis, cell line and cell viability assays and other molecular techniques were employed.
Short description of findings	According to the authors, the study demonstrated that Roundup could impair spermatogenesis, decrease sperm motility and concentration, and increase the sperm deformity rate in mice. In addition, excessive apoptosis of germ cells accompanied by the overexpression of XAF1 occurred after Roundup exposure both *in vitro* and *in vivo*. Furthermore, the low expression of XIAP (X-linked inhibitor of apoptosis) induced by Roundup was inversely correlated with XAF1. Moreover, the knockdown of XAF1 attenuated germ cell apoptosis, improved XIAP expression and inhibited the activation of its downstream target proteins, caspase-3 and PARP, after Roundup exposure. Taken together, our data indicated that XAF1 plays an important role in Roundup-induced male germ cell apoptosis. The study suggested that Roundup exposure has potential negative implications on male reproductive health in mammals.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: In this study, a Roundup formulation containing polyethoxylated tallow amine (POEA) was administered via gavage to adult male mice. The data on the formulation are not relevant for the glyphosate EU renewal as the formulation tested in this study contains polyoxyethylene tallow amine (POEA), which is not permitted for use in formulated herbicidal products in the EU.

Data point	CA 9
Author	Kubsad D. *et al*.
Year	2019
Title	Assessment of glyphosate induced epigenetic transgenerational inheritance of pathologies and sperm epimutations: generational toxicology
Document source	Scientific Reports (2019), Vol. 9, No. 1, pp. 6372
Short description of literature article	Aim of the study was an investigation on the generational actions of glyphosate, as ancestral environmental exposures to a variety of factors and toxicants have been shown to promote the epigenetic transgenerational inheritance of adult-onset disease. Female and male rats of an outbred strain Hsd:Sprague Dawley SD (Harlan) at 70 to 100 days of age were fed ad lib with a standard rat diet and ad lib tap water. Timed-pregnant females on days 8 through 14 of gestation were administered daily intraperitoneal injections of glyphosate (25 mg/kg bw/day dissolved in PBS) (Chem Service, Westchester PA) or dimethyl sulfoxide (DMSO) or phosphate-buffered saline (PBS), as previously described. Twenty-five mg/kg for glyphosate is 0.4% of rat oral LD50 and 50% of the NOAEL and considering glyphosate rapid metabolism approximately twice the occupational exposure 3 5 mg/kg per daily exposure.
Short description of findings	In summary, glyphosate was found to promote the epigenetic transgenerational inheritance of disease and pathology through germline (i.e. sperm) epimutations. Negligible pathology was observed in the F0 and F1 generations, while a significant increase in pathology and disease was observed in the F2 generation grand-offspring and F3 generation great-grand-offspring. Therefore, glyphosate appears to have a low or negligible toxic risk for direct exposure, but promotes generational toxicology in future generations. Observations suggest generational toxicology needs to be incorporated into the risk assessment of glyphosate and all other potential toxicants, as previously described. The ability of glyphosate and other environmental toxicants to impact our future generations needs to be considered, and is potentially as important as the direct exposure toxicology done today for risk assessment.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant because the intraperitoneal route of administration is not appropriate for the human health risk assessment of glyphosate, and is associated with dissimilar toxicokinetics when compared oral dosing. Controls were treated with two different dosing vehicles (PBS and DMSO); further, the data provided clearly show that these two vehicles induced different epigenetic alterations in the 1st and 2nd generations of offspring and, therefore, cannot be considered equivalent. Some controls were dropped from the study due to a "founder effect" related to the induction of obesity, one of the primary endpoints evaluated in this study. This deselection of control animals severely confounds and limits interpretation of the study results. Some animals were excluded from individual assessments (e.g., the age at which puberty was attained); however, the reason/basis for their exclusion was not provided and cannot be determined based on the information provided. None of the DNA methylation patterns or phenotypic changes were shown to be shared by all three generations of offspring (F1, F2, and F3). Additionally, only a limited number of phenotypic changes were reported in the F2 and F3 offspring in the glyphosate treated group and most of these were confounded due to the deselection of controls based on obesity. The expected background ranges for findings were not provided; thus, it is not possible to assess whether the findings observed in the glyphosate group animals were outside the normal expected ranges. Some of the reported data appear to be outside the spontaneous ranges reported by other laboratories (i.e., fertility rates and mean litter sizes) and suggest that the study animals may have been of sub standard health. In addition it is not clear what has been actually tested (glyphosate acid,

glyphosate salt, glyphosate-based formulation). Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Lorenz V. *et al*.
Year	2019
Title	Epigenetic disruption of estrogen receptor alpha is induced by a glyphosate based herbicide in the preimplantation uterus of rats.
Document source	Molecular and Cellular Endocrinology (2019), Vol. 480, pp. 133-141
Short description of literature article	In the present work, the researchers investigated whether perinatal exposure to a glyphosate-based herbicide (GBH) alters the transcriptional regulation of the oestrogen receptor alpha (ERα) gene in the uterus of F1 dams during the preimplantation period. The researchers assessed total ERα mRNA levels and the relative abundance of ERα transcripts with alternative 5′ untranslated regions (5′UTRs). Moreover, the researchers analysed the methylation status and histone post-translational modifications (PTMs) in the regulatory region of the ERα gene as potential epigenetic marks induced by GBH exposure. The glyphosate formulation used in this study was MAGNUM SUPER II marketed in Argentina by Grupo Agros S.R.L. (66.2% of glyphosate potassium salt; equivalent to 54% w/v of glyphosate acid). Pregnant rats (F0) were orally treated with 350 mg glyphosate/kg bw/day through food from gestational day (GD) 9 until weaning. F1 females were bred, and uterine samples were collected on GD5 (preimplantation period). ERα mRNA levels and its transcript variants were evaluated by RT qPCR. Enzyme specific restriction sites and predicted transcription factors were searched *in silico* in the ERα promoter regions to assess the methylation status using the methylation sensitive restriction enzymes-PCR technique. Post translational modifications of histones were studied by the chromatin immunoprecipitation assay.
Short description of findings	GBH upregulated the expression of total ERα mRNA by increasing the abundance of the ERα-O transcript variant. In addition, different epigenetic changes were detected in the O promoter. A decrease in DNA methylation was observed in one of the three sites evaluated in the O promoter. Moreover, histone H4 acetylation and histone H3 lysine 9 trimethylation (H3K9me3) were enriched in the O promoter in GBH exposed rats, whereas H3K27me3 was decreased. All these alterations could account for the increase in ERα gene expression. According to the authors, their findings show that perinatal exposure to a GBH causes long term epigenetic disruption of the uterine ERα gene, which could be associated with the GBH induced implantation failures.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Formulation tested (MAGNUM SUPER II, 66.2% potassium salt, 54% w/v a.e.) is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Meyer Monath M. *et al*.
Year	2014
Title	Development of a multi residue method in a fetal matrix: analysis of meconium
Document source	Analytical and Bioanalytical Chemistry (2014), Vol. 406, No. 30, pp. 7785-7797
Short description of literature article	Meconium is the earliest stool of newborns. It is a complex matrix that reflects the degree of foetal exposure to environmental pollutants. To investigate exposure to xenobiotics, an analytical method was developed to identify and quantify some pesticides and their metabolites and BTEX metabolites in meconium. Samples were prepared by two liquid-solid extractions and purified twice using SPE cartridges, followed by analysis with liquid chromatography coupled with tandem mass spectrometry. SPE cartridges (polymeric phase with hydrophilic and hydrophobic interactions, ion exchange, mixed mode) were tested and matrix effects were evaluated to determine purification performance. The quantification limits in meconium of this multi residue method were in the range of 30 ng/g. The analytical method was applied to "real" meconium samples. Some target analytes were determined in most samples.
Short description of findings	Meconium is a complex matrix that can reflect foetal exposure to micropollutants. A sample preparation method was developed to simultaneously analyse a large range of molecules, from very polar to nonpolar. Despite two SPE purification steps, severe matrix effects were noticed on the LC-MS-MS signal. Because of these matrix effects a matrix match calibration, preferably using internal calibration with labelled compounds, is recommended for the analysis of the meconium matrix. The analysis of meconium samples revealed a significant exposure of the foetus to the studied pollutants. This method will be applied to a total of 235 samples to obtain a more statistically significant set of results. In perspective, the developed multi-residue method could also be used for the detection and quantification of other xenobiotics in meconium.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This is primarily an analytical method paper for determination of multiple analytes (including glyphosate) in meconium. Actual meconium samples were analysed. Minimal details of results provided, and no detections of glyphosate reported, therefore the article is not relevant.

Data point	CA 9
Author	Perego M. C. *et al*.
Year	2017
Title	Influence of a Roundup formulation on glyphosate effects on steroidogenesis and proliferation of bovine granulosa cells *in vitro*
Document source	Chemosphere (2017), Vol. 188, pp. 274-279
Short description of literature article	The purpose of this study was to determine if glyphosate alone (GLPH) or in formulation with Roundup (G RU) can affect granulosa cell proliferation and steroid production. Four experiments were conducted.
Short description of findings	Four experiments were conducted. In Exp. 1, 10 and 300 mg/mL of GLPH had no effect ($P > 0.05$) on cell numbers, estradiol or progesterone production, whereas 10 and 300 mg/mL of G-RU dramatically decreased ($P < 0.05$) cell numbers and estradiol and progesterone production. In Exp. 2, G-RU at 0.1 mg/mL had no significant effect whereas G-RU at 10 mg/mL decreased ($P < 0.05$) GC numbers, progesterone and estradiol production. In the absence of IGF1 but presence of FSH, 1 mg/mL of G-RU decreased ($P < 0.05$) estradiol production, whereas in the presence of IGF1 and FSH, 1 mg/mL of G-RU increased ($P < 0.05$) cell numbers, progesterone and estradiol production. In Exp. 3, IGF1 significantly increased cell numbers (by 2.8-fold) and estradiol (by 17.8-fold) and progesterone (by 6.1-fold) production. GLPH at 10 mg/mL alone had no significant effect on FSH induced (i.e., basal) or FSH plus IGF1 induced cell numbers, estradiol or progesterone production. However, G-RU at 10 mg/mL significantly inhibited FSH plus IGF1-induced cell numbers, estradiol and progesterone production by 65%-91%. In Exp. 4, 48 h treatment of G-RU had no significant effect on viability of attached cells.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This study examines *in vitro* formulation effects only, rather than glyphosate alone. The exact composition of the Roundup formulation tested is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Rappazzo K. M. *et al*.
Year	2019
Title	Maternal residential exposure to specific agricultural pesticide active ingredients and birth defects in a 2003 2005 North Carolina birth cohort
Document source	Birth Defects Research (2019), Vol. 111, No. 6, pp. 312-323
Short description of literature article	Within a North Carolina birth cohort of more than 300,000 singleton births during the years 2003-2005, Rappazzo *et al*. conducted a case control study of seven pesticides (2,4-D, cyhalothrin, glyphosate, mepiquat, metoachlor, paraquat, pendimethalin) and 10 separate birth defects determined through linkage with the North Carolina Birth Defects Monitoring Program: atrial septal defect (n = 1020), patent ductus arteriosus (1364), hypoplastic left heart syndrome (N = 75), tracheal esophageal fistula (n = 75), hypertrophic pyloric stenosis (n = 592), Hirschsprung's disease (n = 77), hypospadias (936), upper (n = 84) and lower limb (n = 39) deficiencies, and choanal atresia (n = 50). Controls were those without birth defects. Exposure was assessed by residential proximity to agricultural cropland within 500 meters and recorded use of specific pesticides on specific crops in 2002 and during the period 2008-2010. The earliest planting and last harvesting dates for each crop defined the probable window of exposure when it overlapped with 1 month before conception through the third month of pregnancy. The sum of presumed pounds applied for each pesticide was used to define extent of exposure. Logistic regression was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for each exposure and type of birth defect. P values were not provided.
Short description of findings	Hypospadias were positively associated with exposures to 2,4-D (OR 50th to <90th percentile: 1.39 [1.18, 1.64]), mepiquat (OR 50th to <90th: 1.10 [0.90, 1.34]), paraquat (OR 50th to <90th: 1.14 [0.93, 1.39]), and pendimethalin (OR 50th to <90th: 1.21 [1.01, 1.44]), but not S-metolachlor (OR50th to <90th: 1.00 [0.81, 1.22]). Whereas atrial septal defects were positively associated with higher levels of exposure to glyphosate, cyhalothrin, S-metolachlor, mepiquat, and pendimethalin (ORs ranged from 1.22 to 1.35 for 50th to <90th exposures, and 1.72 to 2.09 for >90th exposures); associations with paraquat were null or inconsistent (OR 50th to <90th: 1.05 [0.87, 1.27]). The authors concluded that their results suggest differing patterns of association for birth defects with residential exposure to seven pesticide active ingredients in North Carolina.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Highly speculative exposure assessment limited to pesticides makes it impossible to adequately assess results. Therefore this study is not relevant. **Further points for clarification:** This study is not relevant to the ongoing evaluation for glyphosate. Comments rendered here are specific for glyphosate, but likely would apply to the other pesticides as well. The most glaring problem with the study is the extreme inadequacy of the exposure assessment. The (presumed) exposure metric is inconsistent with what is known about glyphosate exposure through biomonitoring of individuals not directly involved in a pesticide application and living on farms at the time of application (Acquavella *et al*. 2004, Niemann *et al*. 2015, Solomon 2016). Also, the exposure assessment is based on patterns of applications that happened during years outside of the study period. The exposure metric piles assumptions on top of assumptions with no validation at any stage. Glyphosate biomonitoring data would suggest strongly that few, if any, of the assigned glyphosate exposures would have been validated had the subjects been biomonitored directly. Indirect exposure metrics of the type used in this study should be validated to some degree before they are used in an epidemiologic analysis. Otherwise, they are just

speculation, likely with extremely high exposure misclassification ratios.
There were also limitations in the analyses in terms of controlling for multiple pesticide and other exposures. Further, the presentation of the results mostly in a graphical format and without p values obscured aspects of the results that would have aided interpretation. Uncertainties about women actually being in their residences or living at the residence of record at the specific times of presumed applications are another source of uncertainty in this study.
It seems that all of the non-glyphosate pesticides were associated with a birth defect in these analyses. Usually, when every exposure has a positive association with the outcome(s), given that all these pesticides have undergone risk assessments are not considered hazardous to the general public, it suggests the study has a marked problem with false positive findings. Arguments about non-differential misclassification are not relevant when the extent of the misclassification is unknown and may be nearly complete. Also, there are likely systematic sources of error in this study that are not appreciated by the authors.
In conclusion, the results of this study are not reliable for making determinations about glyphosate related birth outcomes.

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Data point	CA 9
Author	Romano R. M. *et al.*
Year	2010
Title	Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology
Document source	Archives of toxicology (2010), Vol. 84, No. 4, pp. 309-17
Short description of literature article	This is an *in vivo* experiment conducted with Roundup Transorb on pre pubertal Wistar rats. Evaluations included the progression of puberty, body development, the hormonal production of testosterone, estradiol and corticosterone, and the morphology of the testis.
Short description of findings	Results showed that the herbicide: (1) significantly changed the progression of puberty in a dose-dependent manner; (2) reduced the testosterone production in seminiferous tubule morphology; (3) decreased significantly the epithelium height ($P < 0.001$; control = 85.8 ± 2.8 µm; 5 mg/kg = 71.9 ± 5.3 µm; 50 mg/kg = 69.1 ± 1.7 µm; 250 mg/kg = 65.2 ± 1.3 µm) and increased the luminal diameter ($P < 0.01$; control = 94.0 ± 5.7 µm; 5 mg/kg = 116.6 ± 6.6 µm; 50 mg/kg = 114.3 ± 3.1 µm; 250 mg/kg = 130.3 ± 4.8 µm); (4) no difference in tubular diameter was observed; and (5) relative to the controls, no differences in serum corticosterone or estradiol levels were detected, but the concentrations of testosterone serum were lower in all treated groups ($P < 0.001$; control = 154.5 ± 12.9 ng/dL; 5 mg/kg = 108.6 ± 19.6 ng/dL; 50 mg/dL = 84.5 ± 12.2 ng/dL; 250 mg/kg = 76.9 ± 14.2 ng/dL).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The test material was a glyphosate based formulation Roundup Transorb which is not the EU representative formulation MON 52276, therefore, the article is not relevant. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Romano M. A. *et al*.
Year	2012
Title	Glyphosate impairs male offspring reproductive development by disrupting gonadotropin expression
Document source	Archives of Toxicology (2012), Vol. 86, No. 4, pp. 663-73
Short description of literature article	Sexual behaviour of 60-day-old male rat offspring from females treated with Roundup Transorb during the perinatal period had been assessed. The serum concentrations of testosterone, estradiol, FSH and LH were measured. The pituitary expression of mRNA and protein content of LH and FSH was also analysed to assess the possible glyphosate-mediated interference with their production. Changes in sex hormone serum concentrations may also affect sperm production and the morphology of the seminiferous epithelium, which were evaluated by testicular and epididymal sperm counts and the morphometric analysis of histological sections. The weight of the testes, epididymis and the seminal vesicle; the growth of the animals; and the weight and age at puberty were also recorded to evaluate the effect of the treatment on these parameters.
Short description of findings	This study shows effects on the reproductive development of male offspring from dams treated with Roundup Transorb only in the perinatal period. The authors conclude that the exposure promotes behavioural changes and histological and endocrine problems in reproductive parameters and these changes are reflected by hypersecretion of androgens and increased gonadal activity, sperm production and libido.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: The test material was a glyphosate based formulation Roundup Transorb which is not the EU representative formulation MON 52276, therefore, the article is not relevant. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Stur E. *et al*.
Year	2019
Title	Glyphosate based herbicides at low doses affect canonical pathways in estrogen positive and negative breast cancer cell lines
Document source	PloS one (2019), Vol. 14, No. 7, pp. e0219610
Short description of literature article	The study aimed to identify gene expression changes in ER+ and ER- BC cell lines treated with Roundup Original herbicide formulation (Monsanto, São Paulo, Brazil) and aminomethylphosphonic acid (AMPA), to address changes in canonical pathways that would be related or not with the ER pathway, which the authors believe could interfere with cell proliferation.
Short description of findings	The authors claim that Roundup Original, at much lower doses than the ones used in agriculture, was able to deregulate important intracellular pathways in ER+ and triple-negative BC cell lines. Alterations in gene expression of cell cycle pathways were more prominent on MCF-7 than MDA-MB-468 cells. In contrast, AMPA exposure had a higher effect on MDA-MB-468 than MCF-7 cells.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate as the concentration of AMPA used (10 mM) is beyond the physiologically acceptable range (>1 mM). Evaluation of a glyphosate based herbicide in *in vitro* systems is not relevant to the risk assessment of glyphosate

due to the effects of surfactants on cells. In addition, Roundup Original commercialized in Brazil is not the EU representative formulation for the glyphosate EU renewal.
Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Teleken J. L. *et al*.
Year	2019
Title	Glyphosate-based herbicide exposure during pregnancy and lactation malprograms the male reproductive morphofunction in F1 offspring
Document source	Journal of Developmental Origins of Health and Disease (2019), Vol. 11, No. 2, pp 146-153
Short description of literature article	Female mice consumed 0.5% Roundup Original DI (Monsanto, Brazil) in their drinking water [glyphosate-based herbicide (GBH) group] or filtered water [control (CTRL) group] from the fourth day of pregnancy until the end of the lactation period. Male F1 offspring were designated, according to their mother's treatment, as CTRL-F1 and GBH-F1.
Short description of findings	Female mice that drank GBH reduced body weight (BW) gain during gestation, but no alterations in litter size. GBH male F1 offspring did not exhibit modifications in BW; they, however, demonstrated delayed testicular descent. Furthermore, at PND150, GBH-F1 mice presented a lower number of spermatozoa in the cauda epididymis and reduced epithelial height of the seminiferous epithelium. Notably, intra-testicular testosterone concentrations were enhanced in GBH-F1 mice; the researchers show that it is an effect associated with increased plasma and pituitary concentrations of luteinizing hormone.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This study described a glyphosate-based herbicide dosed to mice. Roundup Original DI (containing POEA) is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Turkmen R. *et al*.
Year	2019
Title	Prenatal and neonatal exposure to glyphosate based herbicide reduces the primordial to primary follicle transition in the newborn rat ovary: a preliminary study
Document source	Kocatepe Veterinary Journal (2019), Vol. 12, No. 2, pp. 168-177
Short description of literature article	This study investigated how a glyphosate based herbicide (GBH), Knockdown 48 SL; Safa Agriculture Inc., Turkey, affects the proportional distribution of ovarian follicles that develop from the 18th day of the embryo period (E18) to the 7th postnatal day (PND7) in newborn female rats. A total of 6 pregnant rats that were used in the study were divided into two groups so that there would be 3 pregnant rats in the control group and 3 pregnant rats in the GBH group. Starting from E21 to E18 the pregnant rats in the experimental group were administered at 50 mg/kg/day GBH subcutaneously and the physiological saline was administered as a vehicle to the control group. Subsequently, female pups received vehicle or 2 mg/kg GBH from PND1 to PND7. On PND8, all-female offspring (neonatal period, 6 newborn female rats from each group) were sacrificed by light ether anaesthesia. For the histological examination of the dissected ovaries, the primordial, primary, secondary and preantral follicle numbers were determined using Crossman's modified triple staining method and Periodic Acid Shiff (PAS) staining methods.
Short description of findings	The percentage of primordial follicles was significantly higher in the ovaries of female rats in the GBH exposed group compare to the control group. However, the percentage of primary, secondary and preantral follicles was lower. Thus, it was observed that prenatal and neonatal GBH exposure decreased the transition of the primordial follicle to the primary follicle.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: Rats gavaged with a glyphosate based herbicide (Knockdown 48 SL; Safa Agriculture Inc., Turkey). The formulation tested is not the EU representative formulation, therefore, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Varayoud J. *et al*.
Year	2017
Title	Effects of a glyphosate based herbicide on the uterus of adult ovariectomized rats
Document source	Environmental Toxicology (2017), Vol. 32, No. 4, pp. 1191-1201
Short description of literature article	The authors evaluated the potential estrogenic effects of a glyphosate based herbicide (GBH) formulation using the uterotrophic assay. Adult ovariectomized rats were subcutaneously injected for 3 consecutive days with: saline solution (vehicle control), 2.10^{-5} g E2/kg/day (uterotrophic dose; UE_2), 2.10^{-7} g E_2/kg/day (nonuterotrophic dose; NUE_2), or 0.5, 5, or 50 mg GBH/kg/day. Twenty four hours after the last injection, the uterus was removed and weighed and processed for histopathology and mRNA extraction. Epithelial cell proliferation and height and expression of oestrogen-responsive genes were evaluated (oestrogen receptors, ERα and ERβ; progesterone receptor, PR; complement 3, C3). Uterine weight and epithelial proliferation were not affected by GBH. However, the luminal epithelial cell height increased at GBH0.5. ERα mRNA was downregulated by all GBH doses and E2 groups, whereas PR and C3 mRNA were diminished by GBH0.5. GBH5-, GBH50-, and UE_2 treated rats showed downregulated ERα protein expression in luminal epithelial cells, while the receptor was upregulated in the stroma. GBH upregulated ERβ (GBH0.5–50) and PR (GBH5) expressions in glandular epithelial cells, a similar effect to that of NUE_2 group.
Short description of findings	The authors claimed that, although the uterine weight was not affected, GBH modulates the expression of oestrogen sensitive genes.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for the risk assessment of glyphosate, as a non EU representative glyphosate formulation was tested (662 mg/mL of glyphosate potassium salt). Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or of one of the other components.

Data point	CA 9
Author	Von Ehrenstein O.S. *et al*.
Year	2019
Title	Prenatal and infant exposure to ambient pesticides and autism spectrum disorder in children: population based case control study
Document source	BMJ (2019), Vol. 364, pp. 1962
Short description of literature article	The authors conducted an autism case control study in California to assess risk from 11 pesticides. Cases were 2,961 individuals with diagnosed autism spectrum disorder. Controls were derived from birth records and matched to cases 10:1 by birth year and sex. Exposure was defined as (ever verses never) proximity of the (geocoded) mother's residence at the birth within a 2,000 meter radius to pesticide applications recorded in the California Pesticide Use Reporting system. Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by multivariable logistic regression, adjusting for selected confounders.
Short description of findings	Risk of autism spectrum disorder was associated with estimated prenatal exposure to numerous pesticides: glyphosate (OR = 1.16, 95% CI 1.06 to 1.27), chlorpyrifos (OR = 1.13, 95% CI 1.05 to 1.23), diazinon (OR = 1.11, 95% CI 1.01 to 1.21), malathion (OR = 1.11, 95% CI 1.01 to 1.22), avermectin (OR = 1.12, 95% CI 1.04 to 1.22), and permethrin (OR = 1.10, 95% CI 1.01 to 1.20). For autism spectrum disorder with intellectual disability, positive associations were reported for glyphosate (OR = 1.33, 95% CI 1.05 to 1.69), chlorpyrifos (OR = 1.27, 95% CI 1.04 to 1.56), diazinon (OR = 1.41, 95% CI 1.15 to 1.73), permethrin (OR = 1.46, 95% CI 1.20 to 1.78), methyl bromide (OR = 1.33, 95% CI 1.07 to 1.64), and myclobutanil (OR = 1.32, 95% CI 1.09 to 1.60). The authors concluded that their results suggest that an offspring's risk of autism spectrum disorder increases following prenatal exposure to ambient pesticides within 2,000 meters of their mother's residential birth address.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is not relevant for the risk assessment of glyphosate with relation to ED because the pathology investigated is not ED related (autism spectrum disorder in children).

Further points for clarification:

This study is not relevant to the ongoing evaluation for glyphosate. Comments herein are specific for glyphosate, but likely would apply to the other pesticides as well.

The major problem with the study is the extreme inadequacy of the exposure assessment. The Farm Family Exposure Study found virtually all spouses who lived on a farm during the time of a glyphosate application did not have detectable glyphosate exposure as assessed by 5-day, 24-hour urinary biomonitoring (Acquavella *et al*. 2004). It is not conceivable, therefore, that a glyphosate application remote from a residence could result in appreciable exposure to a pregnant woman, even assuming the pregnant woman was home during the time of the application. The authors cite some references to support their approach to exposure assessment. But a careful consideration of those references would show they are not relevant to the approach used in this study. Indirect exposure metrics of the type, especially those that extend the exposure corridor up to 2,000 meters (2,500 meters in a sensitivity analysis), should be validated as they are intended to be used before they are employed in an epidemiologic analysis. Otherwise, they may misclassify virtually everyone who is assumed to be exposed.

It is noteworthy that many non glyphosate pesticides were associated with autism in these analyses. Usually, when positive results are many for a study of regulated chemicals, especially a study with questionable exposure methodology, the study is likely to have a marked problem with false positives. The authors' esoteric arguments about non differential misclassification are not relevant when the extent of the misclassification is unknown and may be nearly complete.

There were also limitations in the analyses in terms of controlling for multiple pesticides and other potential confounders. These limitations are worthy of careful consideration, but are a secondary consideration to the limitations of the authors' approach to exposure assessment.

Uncertainties about the birth mothers actually being in their residences or living at the residence of record at the times of presumed pesticide applications are another source of uncertainty in this study. More study of this issue is needed because record linkage pesticide studies of this type are relatively frequent. These uncertainties would be an important consideration regarding exposure misclassification were the exposure assessment approach plausible.

In conclusion, the results of this study are not reliable for making determinations about the relationship between pesticides and autism in California.

References

Acquavella JF, Alexander BH, Mandel JS, *et al*. Glyphosate Biomonitoring for Farmer Applicators and their Families: Results from the Farm Family Exposure Study. Environ Health Perspect 2004, 112:321-326.

Data point	CA 9
Author	Youness E. R. *et al*.
Year	2016
Title	The protective effect of orange juice on glyphosate toxicity in adult male mice.
Document source	Journal of Chemical and Pharmaceutical Research (2016), Vol. 8, No. 3, pp. 13-28
Short description of literature article	This study aimed to investigate orange juice protective role against the toxicity of glyphosate induced in mice. Fifty-six adult male albino mice were divided into seven groups.
Short description of findings	Oral administration of glyphosate caused a significant increase in malondialdehyde (MDA) levels, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, carcino-embryonic antigen (CEA), and DNA damage and decrease in serum testosterone levels. Treatment with orange juice combined with 500 mg/kg body weight of glyphosate for 2 and 4 weeks decreased the incidence of hepatotoxicity, nephrotoxicity, lipid peroxidation, genotoxicity and serum CEA in concomitant with significant elevation in serum testosterone levels compared to glyphosate treated groups. Results showed that orange juice is a potent protector against glyphosate-induced toxicity, and its protective role is time-dependent.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This study uses excessively high gavage doses to rats and is therefore not relevant to glyphosate EU renewal / risk assessment. It is ambiguous if pure glyphosate or a glyphosate-based formulation Roundup was tested. In case a formulation was tested, the exact composition of the Roundup formulation is not stated in the paper (a.i. content, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. The oral gavage (assumed 30 mL/kg) was higher than recommended in the OECD TG 408. No detailed raw data and historical control data were provided. Same control mice were obviously used for all treatment times as only one control group was mentioned and nearly the same results were listed for 2- and 4-week treatments.

Data point	CA 9
Author	Yu H. *et al*.
Year	2013
Title	The antagonistic effects of tea polyphenols on damage of mouse Sertoli cells induced by glyphosate
Document source	Acta Nutrimenta Sinica (2013), Vol. 35, No. 3, pp. 283-87
Short description of literature article	The objective of this work was to observe the protective effect of tea polyphenols (TP) on the damage induced by glyphosate-based herbicide (GBH, 41% isopropylamine hydrochloride) in the Sertoli cells (SC). SC from weaning mice were obtained and cultured. The SC were identified by immune-cytochemical reaction. The damage model of SC was induced by GBH. The cells were divided randomly into control group, GBH-model group and TP protective group (the SC pre-treated with 10, 20, 40, 80, 160 μg/mL TP for 12 h, and then GBH added to SC for 24 h respectively). The cell viability was measured by methylthiazolyl blue tetrazolium (MTT), and the morphological observation of SC was performed under inverted phase-contrast microscope. The cell apoptosis was observed by TdT-mediated dUTP nick end labelling (TUNEL), and their nuclei were observed with Hoechst33342 fluorescent staining. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were measured.
Short description of findings	TP played a protective role against SC damage induced by GBH between the concentration from 10 to 80 μg/mL. TP increased the cell viabilities, SOD and GSH-Px activities. Meanwhile, the cell apoptosis index, the level of MDA and chromatin concentration decreased. The protective effect of TP on the damage of cells could not be observed at 160 μg/mL.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: *In vitro* study testing of what appears to be a formulated product, described as glyphosate (41% isopropylamine hydrochloride, Monsanto) dosed at 10-160 μg/mL (high glyphosate levels of 24-390 μM plus surfactant), well above any potential physiological concentrations in Sertoli cells. Therefore, the paper is not considered relevant to the glyphosate EU renewal. The exact composition of the tested item is not stated in the paper (a.i. content, source, surfactant system / co-formulants). It is therefore not possible to confirm whether the product used is the representative glyphosate formulation MON 52276 relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components. Given this uncertainty over what was tested in this study, the paper is not considered relevant to the glyphosate EU renewal.

Data point	CA 9
Author	Yu N. *et al.*
Year	2018
Title	Circular RNA expression profiles in hippocampus from mice with perinatal glyphosate exposure
Document source	Biochemical and Biophysical Research Communications (2018), Vol. 501, No. 4, pp. 838-845
Short description of literature article	Considering the pivotal role of circular RNAs (circRNAs) in the regulation of gene expression, a circRNA microarray method was used in this study to investigate circRNA expression changes in the hippocampus of mice with perinatal glyphosate exposure.
Short description of findings	This study identified that aberrantly expressed circRNAs are involved in glyphosate-induced neurotoxicity. It also showed that these circRNAs might affect the expression of their target genes by regulating stress associated steroid metabolism pathways. In the future, more investigations are needed to validate the interaction between circRNAs and their target miRNAs and genes. Finally, the roles of circRNAs in glyphosate induced neurotoxicity will be investigated systematically according to the circRNA-miRNA gene network.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This publication is considered not relevant for risk assessment of glyphosate with relation to endocrine disruptors (ED) because a non-ED related endpoint was investigated (circular RNA expression profiles in the hippocampus). The surfactant system of the tested formulation Roundup® (Monsanto Company, St. Louis, MO, USA)marketed in China differs to the EU representative glyphosate formulation MON 52276. Therefore the article is not considered relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Zanardi M. V. *et al*.
Year	2019
Title	Glyphosate-based herbicide induces hyperplastic ducts in the mammary gland of aging Wistar rats
Document source	Molecular and Cellular Endocrinology (2019), Vol. 501, pp. 110658
Short description of literature article	The authors aimed to determine whether early postnatal exposure to a glyphosate-based formulation (GBH, Roundup FULL II) induces long term effects on the rat mammary gland. Thus, female Wistar pups were injected with saline solution (Control) or GBH (2 mg/kg/day) on postnatal days (PND) 1, 3, 5 and 7. At 20 months of age, mammary gland samples were collected to determine histomorphological features, proliferation index and the expression of steroid hormone receptors expression, by immunohistochemistry, and serum samples were collected to assess 17β estradiol (E2) and progesterone (P4) levels.
Short description of findings	Roundup FULL II exposure induced morphological changes evidenced by a higher percentage of hyperplastic ducts and a fibroblastic like stroma in the mammary gland. GBH-treated rats also showed a high expression of steroid hormone receptors in hyperplastic ducts.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: This study examines the effects of glyphosate based herbicide dosed to rats. As Roundup FULL II is not the EU-representative formulation, the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Zhao W. *et al*.
Year	2013
Title	Effects of glyphosate on apoptosis and expressions of androgen binding protein and vimentin mRNA in mouse Sertoli cells
Document source	Journal of Southern Medical University (2013), Vol. 33, No. 11, pp. 1709-13
Short description of literature article	The objective of the study was to investigate the effect of different doses of a glyphosate-based herbicide (41% isopropylamine salt water agent, commercialized by Mengshandu Company) on apoptosis and expressions of androgen-binding protein (ABP) and vimentin mRNA in mouse Sertoli cells. Primarily cultured mouse Sertoli cells incubated with different doses of GBH (60, 90, 120, 150, and 180 mg/L) for 24 h. The growth and morphological alterations in the cells were observed under inverted microscope, and the cell proliferation rate was evaluated with MTT assay. Hoechst 33342 staining was used to detect cell apoptosis after the treatment, and RT-PCR was performed to examine the changes in the expression of ABP and vimentin mRNAs.
Short description of findings	Sertoli cells exposed to a GBH showed a reduced cell volume, cell dissociation with occasional cell disruption. The proliferation of the exposed was suppressed with an increased rate of cell apoptosis and lowered expressions of ABP and vimentin mRNAs ($P < 0.05$).
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: *In vitro* testing of a glyphosate-based herbicide, instead of glyphosate, is not relevant. The tested formulation is not the EU representative formulation thus the article is not relevant to the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

Data point	CA 9
Author	Zhao W. *et al.*
Year	2016
Title	The protective effects of tea polysaccharides on injury and apoptosis of mouse sertoly cells induced by glyphosate
Document source	Current Topics in Nutraceutical Research (2016), Vol. 14, No. 1, pp. 81-90
Short description of literature article	The objective of this study was to investigate the cytotoxic effect of glyphosate-based herbicide Rodeo on mouse Sertoli cells, and to determine if supplementation of tea polysaccharide could ameliorate the effects.
Short description of findings	The results showed that the glyphosate-based herbicide Rodeo inhibited the proliferation of mouse Sertoli cells, increased the level of malondialdehyde (MDA) and dehydrogenase (LDH), and reduced the activity of superoxide dismutase (SOD). Additionally, it was found that glyphosate-based herbicide Rodeo induced Sertoli cells apoptosis and necrosis by Hoechst 33342 assay and flow cytometric analysis, respectively. Administration of tea polysaccharides to Rodeo-treated Sertoli cells reduced the level of MDA and LDH, increased SOD activity, alleviated the proliferation inhibition, and inhibited the apoptosis of Sertoli cells.
Justification as provided in the AIR5 dossier (KCA 9)	Not relevant by full text: *In vitro* testing of a glyphosate-based herbicide is not relevant. In addition, the formulation tested (Rodeo) is not the EU representative formulation and thus the article is not relevant for the glyphosate EU renewal. Furthermore, in the absence of a concurrent control for each of the component of the formulation, it is not possible to conclude whether the observed effects claimed to be secondary to exposure to glyphosate are due to glyphosate exposure or to one of the other components.

B.6.7. NEUROTOXICITY

Refer to separate RAR B.6.7-B.6.10.

B.6.8. OTHER TOXICOLOGICAL STUDIES

Refer to separate RAR B.6.7-B.6.10.

B.6.9. MEDICAL DATA AND INFORMATION

Refer to separate RAR B.6.7-B.6.10.

B.6.10. REFERENCES RELIED ON

Refer to separate RAR B.6.7-B.6.10.