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Australian Pesticides and Veterinary Medicines Authority

A REVIEW OF THE EARTH OPEN SOURCE (EOS) REPORT

"ROUNDUP AND BIRTH DEFECTS: IS THE PUBLIC BEING KEPT IN THE DARK?"

This Report was prepared for the APVMA by

Scitox Assessment Services

Canberra, ACT, Australia

July 2013

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GLOSSARY OF TERMS AND ABBREVIATIONS

AChE	acetylcholinesterase
ADD/ADHD	attention deficit disorder / attention deficit and hyperactivity disorder
ADI	acceptable daily intake
AMPA	aminomethyl sulphonic acid
APVMA	Australian Pesticides and Veterinary Medicines Authority
BNMN	binucleated cells with micronuclei
BVL	(German) Bundesamt fur Verbraucherschutz und Lebensmittelsicherheit
bw	body weight
CFR	conditional fecundability ratio
ChE	cholinesterase
CI	confidence interval
DNA	deoxyribonucleic acid
DoHA	Department of Health and Ageing
DSEWPaC	Department of Sustainability, Environment, Water, Population and Communities
EOS	Earth Open Source
EU	European Union
g	gram
GBHF	glyphosate-based herbicide formulation
GC	gas chromatography
GD	gestation day
GIT	gastro-intestinal tract
g/L	grams per litre
GLP	good laboratory practice
GM	genetically modified
h	hour
ha	hectare
hAR	human androgen receptor
hER	human oestrogen receptor
HC	historical control
HCL	hairy cell leukaemia
IC50	concentration inhibiting a chemical reaction by 50%
IP	intraperitoneal route of administration
IPA	isopropylamino
IV	intravenous route of administration
JMPR	Joint FAO/WHO Meeting on Pesticide Residues
kg	kilogram
km	kilometre
L	litre
lb/gal	pounds per gallon
LC50	lethal concentration to 50% of test cells or animals
LD50	lethal dose to 50% of test animals
LH	luteinising hormone
LOD	limit of detection
LOEL	lowest observed effect level
LOQ	limit of quantification
µg	microgram

M	molar (concentration)
mg	milligram
mg/kg bw/d	milligrams per kilogram bodyweight per day
mL	millilitre
min	minute
mM	millimolar (concentration)
MM	multiple myeloma
mo	month
MRL	maximum residue limit or level
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NEDI	national estimated daily intake
ng	nanogram
NHL	non-Hodgkin's lymphoma
NOAEL	no observed adverse effect level
NOEL	no observed effect level
NTP	US National Toxicology Program
OECD	Organisation for Economic Cooperation and Development
OR	odds ratio
PND	post natal day
PO	oral route of administration
POEA	polyoxyethylene tallow amine
ppb	parts per billion
ppm	parts per million
RA	retinoic acid
RBC	red blood cell
RNA	ribonucleic acid
RR	relative risk
s	second
SCE	sister chromatid exchange
StAR	steroidogenic acute regulatory protein
TTP	time to pregnancy
US EPA	United States Environmental Protection Agency
WHO	World Health Organization
wk	week
yr	year

1. SUMMARY

In June 2011, Earth Open Source (EOS) published an article titled "Roundup and Birth Defects: Is the public being kept in the dark?" in which the organisation made a number of claims about the safety of the herbicide glyphosate and products containing it. These were said to include:

- Developmental malformations affecting the skull, face, brain and spinal cord in frog and chicken embryos at concentrations lower than used in agricultural and garden spraying;
- Endocrine disruption, reproductive toxicity and a range of developmental malformations in humans and experimental animals;
- Damage to DNA and genetic material in laboratory animals, humans a variety of *in vitro* test systems;
- Cancer of the testis in rats, skin cancer in mice, and blood system cancers in humans; and
- Neurotoxicity, including the development of Parkinson's disease in humans.

EOS was also highly critical of the European Union's review of glyphosate (EU, 2002 and 1998); challenged the design, conduct and scientific independence of industry-funded toxicology studies; and questioned some of the scientific principles normally applied to the assessment of hazard and risk from chemicals.

Given the widespread use of glyphosate in Australia for weed control in agricultural, home garden and other settings, the APVMA has investigated[1] the claims made in the EOS article and created this web-based publication to facilitate communication of its findings with the public and other stakeholders. The APVMA has:

- evaluated the key published studies cited in the EOS article together with some newer related publications and archived toxicology studies;
- examined the EU review of glyphosate and compared its findings with those of similar reviews prepared by the Australian DoHA, the US EPA (1993) and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR, 2004a,b);
- assessed the scientific merit of the EOS arguments and the research upon which they are based; and
- considered whether there are implications for the registration of products containing glyphosate in Australia.

The APVMA's findings are summarised in this section of the publication. More detailed evaluations and scientific discussions of each main issue are presented in Sections 2 to 5 and Appendices 1–5.

1.1 The association between glyphosate / glyphosate-based herbicides and developmental malformations

[1] The work was performed by Mark Jenner of Scitox Assessment Services, Canberra, ACT.

As stated by EOS, Paganelli et al (2010) have shown that glyphosate and a glyphosate-based herbicide formulation (GBHF) cause malformations including microphthalmia and microcephaly (abnormally small eyes and head) in toad and chicken embryos. However, the routes of administration (incubation with, or injection into toad embryos, and injection into chicken eggs) are not relevant to humans and other mammals, whose foetuses can only become exposed to chemicals if they are absorbed by their mother and transferred across the placenta from her blood circulation.

In 1996 the APVMA reviewed glyphosate products because of evidence of toxicity to amphibians when applied in and around aquatic areas. Toxicity was attributed to polyethoxylated tallow amine (POEA) surfactants in some glyphosate products. The APVMA consequently strengthened label warnings and restricted the use of glyphosate products around waterways and water bodies to reduce the risk of aquatic contamination (see http://www.apvma.gov.au/products/review/completed/glyphosate_history.php), until less toxic formulations could be developed and registered. Today, over a third of registered glyphosate products contain low toxicity surfactants, and can be used in or around waterways (see http://www.apvma.gov.au/news_media/community/2010-13_glyphosate_au.php). Nevertheless, the APVMA will refer Paganelli's findings to the Department of Sustainability, Environment, Water, Population and Communities (DSEWPaC) for consideration.

Eight developmental toxicity studies with glyphosate in rats and seven in rabbits have been reviewed by pesticide regulatory agencies and scientific organisations including the APVMA, the US EPA, the EU and the JMPR. These and additional studies have also been evaluated by Kimmel et al (2013). The reviews have concluded that at high oral doses, glyphosate causes toxicity to mother rats and foetuses but is not a teratogen (ie, does not cause foetal malformations). The APVMA is satisfied that the German BVL has not misused historical control (HC) data in its evaluations, despite the EOS claim to this effect.

The lowest NOEL for maternal and foetal toxicity in rats was 300 mg/kg bw/d (1000-times the Australian ADI for glyphosate) and the lowest LOEL in foetuses was 1000 mg/kg bw/d. In rabbits, visceral abnormalities including heart dilation and intraventricular septal defect were reported in six of nine developmental toxicity studies. By the most conservative interpretation, these effects were confined to ~~a~~ doses of 450 and 500 mg/kg bw/d. The lowest NOEL for foetal toxicity in rabbits was 100 mg/kg bw/d, or 333-times higher than the Australian ADI. The margins between women's dietary exposure to glyphosate and the NOELs in laboratory animals are even higher; following a dietary survey of pregnant Australian women and analysis of composite food samples they provided, McQueen et al (2012) estimated that maternal dietary exposure to glyphosate is 0.001 mg/kg bw/d. This dose is 0.33% of the ADI, and is also only 5% of the National Estimated Dietary Intake (NEDI) of 0.02 mg/kg bw/d.

Dallegrave et al (2003) have reported skeletal abnormalities in foetal rats whose mothers were treated at 500–1000 mg/kg bw/d during gestation with an herbicide containing 36% glyphosate and 18% POEA surfactant. However, the study has been criticised for reporting deficiencies and anomalies, and its results may have been affected by non-standard methods used to fix and prepare foetuses for skeletal examination (Williams et al, 2012). The APVMA notes that POEA is not a

developmental toxin and has a NOAEL of 300 mg/kg bw/d in foetal rats (Holson, 1990).

The APVMA has investigated EOS' claims that agricultural use of glyphosate is causing adverse reproductive outcomes in exposed human populations. However, the published body of epidemiological research has produced inconsistent, equivocal or weak evidence of reproductive harm. In particular, most epidemiology studies rely on self-reported exposure information, do not measure exposure, and cannot demonstrate causal associations between glyphosate and reproductive harm. Many studies are also affected by confounding variables including exposure to other possible risk factors and the use of, or potential exposure to, other chemicals.

Furthermore, evidence suggests that exposure of glyphosate product users to glyphosate contained in herbicide products is relatively low, possibly due the low dermal absorption rate of glyphosate, which the EU (2002) has estimated to be less than 3%. In a urinary biomonitoring study of American farming families, the maximum absorbed doses from a single mixing / loading / application event were 0.004 and 0.00004 mg/kg bw in the farmers and spouses, respectively (Acquavella et al, 2004 and JMPR, 2004b). These values represent 1.3 and 0.013% of the Australian ADI for glyphosate.

Therefore, the APVMA is satisfied that glyphosate does not pose a risk of developmental toxicity through public or occupational exposure, despite EOS's claim to this effect.

1.2 The association between glyphosate / glyphosate-based herbicides, endocrine disruption and reproductive toxicity

Numerous single- and multi-generation studies have been performed with glyphosate in rats at daily doses of up to 1500 mg/kg. Despite thorough and systematic investigation of relevant parameters, they have yielded no evidence that glyphosate is toxic towards the male or female reproductive systems. No biologically significant effects occurred in a 13-week US National Toxicology Program (NTP) reproduction toxicity study in rats and mice at dietary doses of up to 5000 and 7500 mg/kg bw/d in the respective species. Furthermore, no effects indicative of endocrine disruption have been found in short-term repeat-dose, subchronic and chronic toxicity studies with glyphosate in laboratory animals, and glyphosate has negligible or weak effects on steroid hormone receptors and biosynthesis *in vitro*.

Little reliance can be placed on the study of Yousef et al (1995), which EOS claims to have demonstrated sperm damage in rabbits. When administered for six weeks, glyphosate may have caused fully or partially reversible decreases in ejaculate volume and the viability and activity of sperm, but the study used low numbers of animals and deficient experimental methods, was markedly affected by variation within the control group, and was poorly reported. It is even unclear what doses of glyphosate were administered.

Although EOS has described glyphosate as causing testicular cancer in rats, independent assessments of the relevant study (Lankas et al, 1981) by Australia, the WHO and the US EPA have concluded that the tumours were not treatment-related. Furthermore, neither testicular tumours nor other forms of cancer have developed in eight other carcinogenicity studies with glyphosate in mice or rats, respectively at doses of up to *ca* 5000 and 1200 mg/kg bw/d. Mink et al (2012) have reviewed the epidemiological literature (7 cohort studies and 14 case-control studies) to evaluate

whether exposure to glyphosate is associated causally with cancer risk in humans. They found no consistent pattern of positive associations to indicate a causal relationship between total cancer (in adults or children) or any site-specific cancer and exposure to glyphosate. This provides strong evidence that glyphosate does not pose a carcinogenicity hazard to humans.

The APVMA anticipates that glyphosate's potential to cause endocrine disruption will be clarified in the near future, as the active has been tested according to US EPA Series 890 Test Guidelines following its selection for Tier 1 screening under the EPA's Endocrine Disruption Screening Program. As at June 2013, all data have been received by the EPA and are currently under review (see http://www.epa.gov/scipoly/index.html). So far three abstracts have been published, demonstrating a lack of potential to interact with oestrogen and androgen receptors in vitro, inhibit steroidogenesis in vitro, affect thyroid-mediated developmental endpoints in the amphibian metamorphosis assay, or cause endocrine disruption in the Hershberger and uterotrophic assays in rats (Levine et al 2012, Webb et al 2012, Saltmiras and Tobia 2012).

There is experimental evidence in support of EOS's assertion that glyphosate-based herbicide formulations (GBHFs) cause reproductive toxicity in drakes and, in male rats, interfere with the maturation of the reproductive organs during puberty. In some studies (Oliviera et al 2007, Romano et al 2010) GBHFs were administered directly to the test animals, while other studies (Dallegrave et al 2007, Romano et al 2012) involved maternal exposure to GBHFs during pregnancy and/or lactation. However, the observed effects have been inconsistent, including increases and decreases in blood testosterone levels and sperm production, and delaying and hastening of the onset of puberty. Furthermore, most of the relevant studies are deficient in aspects of their design and reporting, have used novel, unvalidated test methods, and/or may have been subjected to interference by experimental artefacts. None of the studies have identified which component(s) of the test GBHFs caused the reported effects.

In vitro, some GBHFs have caused anti- androgenic and oestrogenic activity, changes in the expression of hormonally-regulated genes, inhibition of aromatase (an enzyme that converts testosterone to oestradiol), cell injury and death. However, many *in vitro* studies have used cancer cells or other novel test systems, and a 2009 Canadian PMRA assessment concluded that their findings are not representative of the exposure of live animals and humans (see http://www.hc-sc.gc.ca/cps-spc/pubs/pest/_fact-fiche/glyphosate/reconsideration-reexamen-eng.php). Furthermore, surfactants (including POEA) are a likely cause of cellular toxicity and interference with *in vitro* assays of hormonal regulation. Few studies have identified or controlled for the surfactants and other adjuvants present in test formulations, creating uncertainty as to which chemicals are causing the reported effects, and their mode of action.

Therefore, the APVMA believes it is premature to characterise GBHFs as endocrine disruptors.

1.3 Evidence for the genotoxicity and carcinogenicity of glyphosate / glyphosate-based herbicides

Only a small minority of the genotoxicity studies with glyphosate and GBHFs have yielded positive findings, some of which were inconsistent with negative results in other studies examining the same end-point. Interpretation of several published studies is hindered by methodological failings or inadequately-detailed reporting.

Many instances of positive findings could also be explained by cytotoxicity, ie, generalised toxicity against the test cells, tissues or organs, as opposed to direct effects on genetic material. When the activity of glyphosate and GBHFs was compared under the same experimental conditions, the active constituent was usually inactive or much less active than the formulations. Where studies were performed with GBHFs without examining the individual ingredients, it is unknown whether the findings were caused by glyphosate, surfactants or other adjuvants, or depended on interaction between the various formulation components. A recent, comprehensive review of published and sponsored regulatory genotoxicity studies (Kier and Kirkland, 2013) has concluded that glyphosate and typical GBHFs do not appear to present significant genotoxic risk under normal conditions of human or environmental exposures. Studies of genetic injury within human populations have not yielded consistent evidence of a causal association between glyphosate exposure and genotoxicity. Therefore, weight and strength of evidence supports the view that glyphosate is not genotoxic.

Between them, the Australian DoHA, the US EPA, the EU and the JMPR have reviewed four dietary carcinogenicity studies with glyphosate in mice and six similar studies in rats, performed over dose ranges of 11 – *ca* 5000 and 4 – *ca* 1200 mg/kg bw/d in the respective species. Although the incidence of testicular tumours was increased in glyphosate-treated rats in one study (Lankas, 1981), the reviewing agencies agreed that by reference to HC data, the tumours were not related to treatment. Furthermore, tumours did not develop in the testis – or any other organs or tissues – in the remaining carcinogenicity studies.

A GBHF has been found to promote skin tumours when applied dermally to mice at 25 mg/kg bw/d (George et al, 2010), but carcinogenesis depended on prior treatment with a tumour initiator chemical, without which there was no development of cancer. The study did not demonstrate which component(s) of the product caused the promoting activity. The finding is of limited relevance to persons preparing GBHFs for use because the tumour promoting activity was relatively weak, and to achieve an equivalent level of exposure, operators would have to be exposed three times weekly for over a decade at doses unattainable while wearing the required protective clothing and equipment.

The Australian DoHA (2005) and the JMPR (2004b) have assessed nine epidemiological studies performed from 1999 onwards, including those cited by EOS as showing associations between glyphosate and blood system cancers. Some researchers have found increased odds of developing non-Hodgkin's lymphoma, multiple myeloma or hairy cell leukaemia in persons who have used or been exposed to glyphosate. However, the evidence has been inconsistent both between and within studies, whose outcomes are potentially confounded by inaccurate exposure data and exposure to other pesticides and environmental agents. A recent review (Mink et al, 2012) of epidemiological studies relevant to cancer end-points considered seven cohort studies and 14 case—control studies; there was no consistent pattern of positive associations to indicate any causal relationship between total cancer (in adults or children) or any site-specific cancer and exposure to glyphosate.

Currently, the weight and strength of evidence does not support the conclusion that glyphosate causes cancer in either laboratory animals or humans.

1.4 Neurotoxicity of glyphosate / glyphosate-based herbicides

Glyphosate does not have the same biological properties as organophosphate insecticides, and an extensive battery of neurotoxicology and general toxicity studies in laboratory animals has found no evidence that glyphosate inhibits cholinesterase activity, or causes neuropathy or other disorders in the nervous system. The largest and most comprehensive study of pesticide applicators (Kamel et al, 2007) has found no association between the use of glyphosate and Parkinson's disease.

1.5 Human exposure to glyphosate

During the assessment process for pesticides that may leave residues in food, chemicals are assigned an Acceptable Daily Intake (ADI), which is the level of intake of a chemical that can be ingested daily over a lifetime without any appreciable risk to health.

The current ADI for glyphosate, set by the Australian DoHA in 1985, is 0.30 mg/kg bw/d, based on a NOEL of 30 mg/kg bw/d (the highest administered dose) in a three-generation reproduction study in rats. There is a 100-fold safety factor between the pivotal NOEL and the ADI, comprised of a ten-fold component to account for extrapolation from animals to humans and a further ten-fold component to account for variation in sensitivity within the human population. The toxicological studies cited by EOS do not demonstrate any need to revise the ADI.

By comparison with the ADI, the actual level of exposure for Australians is probably much lower. Based on the consumption of food commodities for which the APVMA has set Maximum Residue Limits (MRLs), the National Estimated Daily Intake (NEDI) of glyphosate is 0.02 mg/kg bw/d, or only six percent of the ADI. Even this value may be conservative. Following a dietary survey of pregnant Australian women and analysis of composite food samples they provided, McQueen et al (2012) have estimated that maternal dietary exposure to glyphosate is 0.001 mg/kg bw/d. This dose is 0.33% of the ADI, and is also only 5% of the NEDI of 0.02 mg/kg bw/d. Internationally, the JMPR (2004a) estimated theoretical maximum daily intake for glyphosate is 1% of the WHO ADI of 0–1.0 mg/kg bw/d.

Evidence suggests that exposure of glyphosate product users is also relatively low. This may be due to the relatively low dermal absorption rate, which the EU (2002) assessment estimated to be less than 3% for glyphosate and no more than 1% for glyphosate trimesium. In a biomonitoring study of American farming families, Acquavella et al (2004) detected glyphosate in the urine of 60% of farmers, 4% of their spouses and 12% of their children on the day of application. According to the JMPR (2004b) assessment, the maximum systemic (absorbed) doses from a single mixing / loading / application event were 0.004, 0.00004 and 0.0008 mg/kg bw in the farmers, spouses and children, respectively. These values represent 1.3%, 0.013% and 0.27% of the Australian ADI for glyphosate.

1.6 Overseas assessment activity

The US EPA and the Canadian PMRA initiated routine scheduled re-registration reviews of glyphosate in 2009 and 2010, respectively. Both these regulators will use the reviews to consider new research on glyphosate, relating to potential effects on environmental and human health. The EPA will assess studies on the immunotoxicity and acute and subchronic neurotoxicity of glyphosate, the ecotoxicity of products containing the surfactant POEA, and the ecological risk posed by aminomethyl

phosphonic acid (AMPA, a degradation product of glyphosate). The review is scheduled for completion in 2015 (US EPA, 2009). In addition to the re-registration review, the EPA is also evaluating glyphosate under the Endocrine Disruptor Screening Program. The Canadian review, targeted for completion in 2014, will include health and environmental risk assessments of the POEA/glyphosate combination (see http://www.hc-sc.gc.ca/alt_formats/pdf/pubs/pest/decisions/rev/rev2010-02-eng.pdf).

Conclusions

1. The APVMA currently has no data before it suggesting that glyphosate products registered in Australia and used according to label instructions present any unacceptable risks to human health, the environment and trade.
2. The weight and strength of evidence shows that glyphosate is not genotoxic, carcinogenic, or neurotoxic.
3. Glyphosate causes malformations in toad and chicken embryos treated by incubation and/or injection, but these findings are not predictive of a developmental hazard to humans because of the routes of administration used. Studies in birds and/or rats have reported that some glyphosate-based herbicide formulations (GBHFs) cause foetal skeletal abnormalities, toxicity to the male reproductive system and interference with the maturation of the male reproductive organs during puberty. However, the relevant studies were affected by flawed design, methodology and / or reporting, and the claimed effects on puberty have been inconsistent in different studies.
4. Glyphosate is not a teratogen in rats and rabbits treated via oral administration and has not shown reproductive toxicity in multi-generation dietary studies in rats. Epidemiological studies have found no consistent or convincing evidence of reproductive dysfunction in human populations reportedly exposed to glyphosate. Glyphosate is therefore extremely unlikely to cause reproductive or developmental toxicity in humans under normal conditions of exposure.
5. The potential for glyphosate to cause endocrine disruption will be clarified by the current review under the US EPA's Endocrine Disruptor Screening Program. In studies published so far, glyphosate has shown a lack of activity in the Hershberger and uterotrophic assays in rats or in tests for interaction with oestrogen and androgen receptors, inhibition of steroidogenesis, or interference with metamorphosis in amphibians. At present, there is no scientific justification for classifying glyphosate as an endocrine disruptor.
6. Surfactants present in the test GBHFs may have confounded the results of *in vitro* studies of their effects on hormonal regulation and cellular toxicity. Furthermore, the relevance of some test systems to human hazard and risk assessment is unproven.
7. Most studies with GBHFs have not identified which of their chemical constituents caused the reported effects on cells and laboratory animals, or characterised their mode of action.
8. The toxicological studies cited by EOS do not demonstrate a need to revise the current Australian ADI of 0.3 mg/kg bw/d for glyphosate. The available evidence indicates that there are very wide margins between the ADI and the

actual intake of glyphosate via food and from exposure while preparing and applying glyphosate products.

9. The APVMA will monitor the US and Canadian reviews of glyphosate and consider any new information that emerges.

2. MAIN BODY OF THE REVIEW

2.1 The association between glyphosate / glyphosate—based herbicides and developmental malformations

2.1.1 Effects in toad and bird embryos

According to EOS, Roundup causes developmental malformations in toad and chicken embryos at doses "much lower than those used in agricultural spraying" and "ten times lower than the MRL". These claims are based on an article by Paganelli et al (2010; see Appendix 3), who treated African clawed toad (*Xenopus laevis*) embryos with glyphosate (360 or 500 pg by intracellular injection) or a 480 g/L Roundup formulation (present in the incubation medium at a 5000-fold dilution, or 96 mg glyphosate/L). The test compounds decreased the expression of genes that regulate embryonic development, impaired the formation of neurons (nerve fibres) and the neural crest, and also caused microphthalmia and microcephaly (abnormally small eyes and head).

Incubation with Roundup at 4000- and 3000-fold dilutions caused increases in retinoic acid (RA) signalling activity within toad embryos, whereas co-treatment with a RA-receptor antagonist blocked increases in RA signalling and prevented microcephaly. The study authors also found that injecting Roundup into chicken eggs (20 µL of 3500- or 4500-fold dilutions, equivalent to 2.7 or 2.1 µg glyphosate/egg) caused microphthalmia and microcephaly in the embryos. However, they did not investigate whether the malformations occurred in response to stimulation of RA signalling, as in *Xenopus*.

APVMA comment

Retinoic acid, a metabolite of vitamin A, has a pivotal role in the development of the central nervous system and causes microcephaly, microphthalmia and neural tube defects including spina bifida when administered in excess to pregnant laboratory animals (Maden, 2002). Therefore, in principle, Paganelli's study suggests that glyphosate and glyphosate-based herbicides may have the potential to cause developmental malformations by a mechanism involving RA.

However, caution should be exercised in extrapolating from findings in amphibians and birds to predicting risks for humans. The absorption, distribution, excretion and toxicokinetics of chemicals in pregnant mammals are fundamentally different to those in organisms whose development occurs in the external environment. Furthermore, the experimental routes of administration used by Paganelli (incubation or injection) do not reflect the likely routes of human exposure (oral, dermal, or inhalational) or the protective effect of the placental barrier (BVL, 2010).

Above all, as discussed later in this Section, glyphosate has been tested in numerous developmental studies over a 20—year period in rats and rabbits without causing malformations of the head and neural tube, even at doses high enough to be toxic to the mother and foetus.

2.1.2 Effects in laboratory animals

The major theme of the EOS article is that glyphosate has shown teratogenic activity in industry-sponsored developmental toxicity studies in rats and rabbits, with effects on foetuses including mortality, reduced ossification (bone formation) and increased incidences of skeletal and visceral abnormalities. Furthermore, EOS claims that these findings were wrongly dismissed in the EU (1998) review of glyphosate performed by the German Bundesamt fur Verbraucherschutz und Lebensmittelsicherheit (BVL). In particular, EOS criticises the use of historical control (HC) data to assist in deciding whether foetal malformations and anomalies were related to treatment, or occurred by chance. EOS was concerned that HC data introduced variability into the analysis and obscured the teratogenic effects of glyphosate.

EOS's comments on the BVL evaluations of specific studies can be summarised as follows:

- The BVL evaluation of Tasker et al (1980a) did not consider that an increased incidence of foetal malformations in rats at the highest dose (3500 mg/kg bw/d) was treatment-related, because the incidence lay within the HC range. EOS regards this as unjustifiable, due to the findings of malformations in other studies with glyphosate. EOS also criticises the BVL's definition of sternebral unossification as a variation, rather than a malformation.

- EOS disagrees with the BVL assessment of Suresh (1993a), a developmental study in rabbits at 20, 100 and 500 mg/kg bw/d in which there was an increase at all doses in major visceral anomalies, including dilated heart. Suresh concluded that the NOEL for maternotoxicity was 20 mg/kg but there was no NOEL for foetal visceral malformations. The BVL dismissed the biological significance of the foetal findings, and set the NOEL at 100 mg/kg bw/d based on comparison with HC data.
- The BVL evaluation of Brooker et al (1991b; a gavage study in rabbits at 50, 150 and 450 mg/kg bw/d) was criticised for dismissing an increased incidence of foetal heart malformations at the high dose by reference to HC data.
- EOS criticises the BVL's assessment of Bhide and Patil (1989; a developmental study in rabbits at 125, 250 and 500 mg/kg bw/d), which assigned a NOAEL of 250 mg/kg for developmental toxicity based on embryo- and foetal lethality and visceral and skeletal malformations at the high dose. EOS believes that heart, lung and kidney malformations were increased at all doses, while rudimentary 14^{th} rib was increased at 250 and 500 mg/kg.
- EOS does not concur with the BVL evaluation of an anonymous (1981) oral feeding study in rabbits, in which increased foetal mortality at 50.7 and 255 mg/kg bw/d was not attributed to treatment because the doses were "far below those at which foetal effects were found in the gavage studies."

APVMA comments

The mammalian toxicology of glyphosate has been reviewed by several national and international pesticide regulatory agencies and scientific organisations, including the

APVMA[2], the US EPA, the EU and the Joint FAO/WHO Meeting on Pesticide Residues (JMPR). Between them, these agencies have evaluated eight developmental toxicity studies with glyphosate in rats and seven in rabbits. Kimmel et al (2013) and Williams et al (2012) have also reviewed developmental studies with glyphosate in laboratory animals.

2.1.2.1 Effects in rats

The German BVL assessed six rat developmental studies for the EU and/or JMPR. These are summarised in Appendix 1. There was a wide span of doses, ranging from 22 to 3500 mg/kg bw/d. According to the BVL, maternotoxicity was seen as clinical signs and reduced bodyweight gain at ≥1000 mg/kg, with maternal deaths at 3500 mg/kg. Effects on foetuses comprised increased incidences of wavy ribs, unossified sternebrae[3], and incompletely ossified finger / toe bones, cranial centre and vertebral arches at ≥1000 mg/kg; with increased mortality and depressed litter and mean foetal bodyweights at 3500 mg/kg. Overall, the lowest NOEL for maternal and foetal effects in rats was 300 mg/kg bw/d, a dose 1000-times higher than the Australian ADI for glyphosate.

After closely examining the German evaluations for the EU and JMPR, the APVMA supports the BVL's conclusions, including those relying on HC data[4]. Indeed, it is possible to rebut EOS's claim that the BVL incorrectly dismissed the treatment-relatedness of dwarfism and bent tail seen at 3500 mg/kg bw/d in Tasker et al (1980a). The US EPA, Australian DoHA and Kimmel et al (2013) have also evaluated this study, and independently reached the same conclusions as the BVL. The DoHA (1985) attributed the malformations to genetic factors because all dwarf foetuses were in one litter, all those with bent tails were confined to another litter, and the control and 3500 mg/kg groups had the same number of litters with malformed foetuses.

[2] Human health risk assessments are performed for the APVMA by the Department of Health and Ageing (DoHA).

[3] According to the OECD (2008) Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment, there is no generally accepted classification of malformations (permanent structural changes that may adversely affect survival, development or function) and variations (divergence beyond the usual range of structural constitution, which may not adversely affect survival or health). The nomenclature used by study laboratories and regulatory agencies may therefore vary, in part because there is a continuum between normal and abnormal development, because some observations are classified as malformations in one species and variations in another, or due to the use of different nomenclature conventions by different organisations. The highly authoritative DevTox website (http://www.DevTox.org), whose terminology and classification system was developed by a series of international harmonisation workshops, does not classify sternebral unossification as either a malformation or variation.

[4] Besides identifying the effects of the test compound on animals, the major purpose of regulatory toxicology studies is to establish the doses at which the effects do or do not occur. This is most commonly done by comparing findings from groups of animals treated over a range of doses with those from an untreated group of the same species and genetic background, housed under the same conditions as the test groups. These untreated animals are usually referred to as "study" or "concurrent" controls. In addition to presenting data from the test groups and study controls, reports may also include "historical control" (HC) data from other studies performed in animals from the same supplier and genetic background at the same laboratory. HC mean values and ranges are sometimes used during evaluation to clarify the biological significance of differences between the study controls and groups of animals treated with the test compound. HC data can also provide information about whether a study control group's results are atypical compared with those observed in other control groups. The use of HC data generated within a five-year span around the study under review is accepted internationally under the OECD (2008) guidelines.

Furthermore, based on the available evaluation reports, neither dwarfism nor bent tail occurred at any dose in the other rat studies, or in rabbits.

However, there are possibly significant findings in Dallegrave et al (2003), a developmental toxicity study in which pregnant rats were dosed orally from GD 6–15 with a Roundup formulation containing 360 g/L glyphosate and 18% w/v polyethoxylated tallow amine (POEA)[5]. The doses were equivalent to 500, 750 or 1000 mg glyphosate/kg bw/d. Based on increased mortality in dams at the highest dose, the apparent NOEL for maternotoxicity was 750 mg/kg bw/d but this is uncertain because Dallegrave et al did not report clinical signs, even in dams which died. The test formulation did not affect foetal survival or growth, but from 500 mg/kg upwards caused skeletal abnormalities including ossification deficits, absent and wavy ribs, absent vertebrae, and divided sternebrae and supraoccipital and interparietal bones.

The fact that the test formulation caused malformations at half the lowest foetal LOEL in rat studies with glyphosate active constituent (1000 mg/kg bw/d; see above) suggests that formulation adjuvants caused or contributed to the effects. When Holson (1990) administered POEA to pregnant rats by gavage on GD 6–15 at 15, 100 and 300 mg/kg bw/d, there was significant maternal toxicity at 300 mg/kg while decreased food consumption and mild clinical signs occurred in dams at 100 mg/kg. The maternal NOEL was 15 mg/kg bw/d. Foetal growth and development were unaffected, so the NOEL for developmental toxicity was 300 mg/kg bw/d. In Dallegrave et al (2003), by comparison, rat dams were exposed to POEA in the test formulation at *ca* 250, 375 or 500 mg/kg bw/d, exceeding the maternal LOEL of the pure surfactant by 2.5 to 5-fold. Furthermore, Dallegrave's mid and high dose dams received more POEA than administered in Holson's study (Williams et al 2000; Williams et al, 2012).

Williams et al (2012) have also noted anomalies in the numbers of foetuses, corpora lutea and implantations reported by Dallegrave et al (2003), and commented that Dallegrave used a non-standard method for fixing and protein-digesting foetuses prior to skeletal examination, which may have created areas that appeared to be incompletely ossified. Given the reporting and methodological issues identified in Dallegrave et al (2003), and because there are no other known developmental toxicity studies with GBHFs that can be compared with Dallegrave's study, the APVMA can not reach any further conclusions on Dallegrave's findings.

2.1.2.2 Effects in rabbits

Six of the nine known developmental studies with glyphosate in rabbits have been assessed by the German BVL for the EU and/or JMPR. Two other sponsored regulatory studies have been assessed by Kimmel et al (2013), and a further study (Stauffer Chemical Co, 1983b) was evaluated by the Australian DoHA. The doses spanned from 10 to 500 mg/kg bw/d. Evidence of maternotoxicity was fairly consistent between studies, but the threshold doses for each effect varied widely. Clinical signs and bodyweight depression occurred at $\geq$40 mg/kg, with increased maternal mortality and abortion at $\geq$100 mg/kg and decreased food consumption and

[5] POEA (also known as polyoxyethylene tallow amine and polyoxyethyleneamine; CAS Registry no. 61791-26-2) is a mixture of polyethoxylated long chain alkylamines synthesised from animal-derived fatty acids (Williams et al, 2000).

bodyweight gain at ≥150 mg/kg. Due to the varying LOELs, maternal NOELs lay between 20–250 mg/kg bw/d.

Four gavage studies did not demonstrate any effects on foetuses at the highest doses administered (100 mg/kg bw/d in Stauffer Chemical Co, 1983b; 300 mg/kg in Hojo, 1995; 350 mg/kg in Tasker et al, 1980b and 400 mg/kg in Coles and Doleman, 1996). In four gavage studies there was fetotoxicity, seen as bodyweight depression and reduced skeletal ossification at 300 mg/kg, increased mortality at ≥450 mg/kg and extra 13^{th} rib or unilateral 14^{th} rib at 500 mg/kg bw/d.

Visceral abnormalities occurred in six studies. These included heart or ventricular dilation and cardiomegaly, the incidences of which were elevated at 20, 100 and 500 mg/kg bw/d in Suresh (1993a). By reference to HC data, the BVL concluded that the effects were biologically significant only at the high dose, and set the foetal NOEL at 100 mg/kg bw/d. Intra-ventricular septal defect (either alone or combined with other cardiac abnormalities) was reported in Brooker et al (1991b), Bhide and Patil (1989), Hojo (1995) and Moxon (1996). Brooker et al observed incidences of 3.6% and 5.3% at 150 and 450 mg/kg bw/d, compared with 0.6% among study controls. However, given that the incidences lay within the HC range (0.7–5.9%), the BVL did not ascribe the finding to treatment at either dose. Septal defect was increased at 125, 250 and 500 mg/kg bw/d in Bhide and Patil (incidences were 0.9, 0.8 and 2.6% vs zero among controls). The BVL evaluator reasoned that the finding was unlikely to have been caused by glyphosate but could not exclude a relationship to treatment at 500 mg/kg bw/d. The APVMA concurs with this view, especially in the absence of HC data from the study laboratory. Also in Bhide and Patil, but no other study, there were elevated incidences of absent kidney (0.9, 1.8, 1.6 and 7.7% at 0, 125, 250 and 500 mg/kg bw/d) and postcaval lung lobe (0, 0.9, 1.6 and 5.1% in the respective groups). Again, the BVL attributed the findings to treatment at 500 mg/kg but not at lower doses.

Hojo (1995) reported one foetus affected by interventricular septal defect and hypoplasia of the pulmonary artery at 100 mg/kg bw/d, but no cardiac abnormalities at 10 or 300 mg/kg. Coles and Doleman (1996) observed a foetus with a heart and great vessel defect at 200 mg/kg bw/d but no cases at 50 or 400 mg/kg. Moxon (1996) found three foetuses having heart defects involving septation, one each at 0, 100 and 300 mg/kg bw/d. In these latter three studies, it is clear that the cardiovascular abnormalities were unrelated to treatment.

Overall, the range of foetal NOELs in rabbits was 100–400 mg/kg bw/d, overlapping the lowest foetal LOEL of 300 mg/kg bw/d. The margin between the *lowest* foetal NOEL and the Australian ADI is 333. Examining the dose-effect relationship in the rabbit gavage studies, the most sensitive end-points are foetal bodyweight and skeletal ossification, which were depressed at 300 mg/kg. If cardiac dilation, ventricular septal defect and major visceral malformations (including missing lung lobes and kidney) were indeed caused by glyphosate, by any reasonable interpretation they are confined to the 450 and 500 mg/kg groups. The margin between the doses causing these effects and the Australian ADI is 1500.

The final issue in rabbits involves a seriously-deficient study report of increased foetal deaths occurring at 50.7 and 255 mg/kg bw/d in a developmental study by dietary administration (Anon, 1981). The BVL assigned a NOEL of 10.5 mg/kg bw/d but highlighted the inconsistency between these particular findings and the results in the gavage studies, in which foetal mortality was not enhanced below 300 mg/kg bw/d.

Based on the comparative weight and strength of evidence, this comment is entirely reasonable.

2.1.3 Epidemiological evidence

According to EOS, a report commissioned by the state government of Chaco, Argentina (CPICA, 2010), found an increase of nearly four-fold in the rate of malformations over a decade, coinciding with the expansion of agriculture into the region and a corresponding rise in the use of agrochemicals, including glyphosate.

EOS, Paganelli et al (2010) and Carrasco (2011) cite Benitez-Leite et al (2009) as finding that Paraguayan women exposed to herbicides during pregnancy were more likely than unexposed women to deliver offspring with malformations. These included microcephaly or anencephaly (small head or absence of a cranium), facial defects, myelomeningocele (protruding brain), cleft palate, synotia (ears extended below the jaw), polydactyly (too many fingers / toes) and syndactyly (fused digits). The specific risk factors identified were living near treated soy fields, dwellings located <1 km from treated fields, storage of pesticides in the home, and contact with pesticides (Carrasco, 2011).

EOS also claims that Savitz et al (1997) found high levels of premature births and miscarriages in female members of Canadian farming families that used pesticides, including glyphosate.

APVMA comments

According to the BVL (2010), Mulet (2011) and Saltmiras et al (2011), the database studied by Benitez-Leite et al was small and confined to children born in one hospital. Benitez-Leite et al suspected a relationship between malformations and pesticide (*not specifically herbicide*) exposure but did not provide evidence of maternal exposure to glyphosate, or even mention glyphosate in their article. The association between "living near treated fields" and congenital malformations was weak, with an odds ratio (OR) $1/6^{th}$ of the reported association between malformations and pesticide storage at home.

The "Ontario Farm Family Health Study" (Savitz et al, 1997) has been assessed by the JMPR (2004b), the Australian DoHA (2005), Mink et al (2011) and Williams et al (2012). In a cross-sectional study of 1898 couples and 3984 pregnancies, Savitz et al examined the association between pregnancy outcome and the father's exposure to pesticides during the three months before conception. The study relied on mail questionnaires, with telephone interviews of non-respondents. Couples were asked to provide information on all pregnancies (of which over $1/3^{rd}$ had occurred over 10 years previously) and farm activities and pesticide use over the previous five years. Not all reports of adverse pregnancy outcomes were confirmed from medical or other records, and the study was uncontrolled for maternal age, smoking and previous history of spontaneous abortion.

There were no statistically significant associations with the use of glyphosate alone. There were slightly increased odds ratios (OR) but no statistically significant associations between miscarriage and paternal use of herbicides *and* glyphosate on crops (17 exposed cases, OR = 1.5; 95% Confidence Interval = 0.8–2.7) or in the yard (13 exposed cases, OR = 1.4; 95% CI = 0.7–2.8). Based on five exposed cases, the

OR for pre-term delivery and use of herbicides *and* glyphosate on crops was 2.4 but the risk estimate was of low precision (the 95% CI was 0.8–7.9). There was no association between the use of farm chemicals and small-for-gestational age births or sex ratio.

DoHA questioned the apparent association between miscarriage and herbicide/glyphosate application due to the small number of cases and the imprecision of the risk estimate, noted that the study authors had not directly tested for association between glyphosate use and reproductive effects, and observed that the study was further weakened by the lack of quantitative exposure assessment and data on the time spent using pesticides. The JMPR assessment commented that the claimed associations were weak, were not controlled for confounding factors including other pesticides, and did not meet generally accepted criteria for determining causal relationships.

Sanin et al (2009) undertook a retrospective cohort study of time to pregnancy (TTP) among 2592 fertile women living in five regions of Colombia, between which there was variation in the use of glyphosate-based herbicides. Glyphosate was not used in the region with lowest risk of prolonged time to pregnancy (TTP). The region with greatest risk (fecundability[6] OR of 0.15; 95% CI = 0.12–0.18) was a sugar cane-growing district with a prolonged history of use of glyphosate and other chemicals. Glyphosate was applied to illegal crops in two of three other regions with enhanced risk, but not in the third, an organic agriculture area. The study authors concluded that the observed differences in TTP remained unexplained.

Numerous other epidemiological studies have examined datasets for associations between glyphosate and adverse reproductive outcomes, but found little evidence that glyphosate is causing ill health within human populations. Furthermore, many of these studies are weakened by shortcomings including survey methods prone to inaccurate or biased recall of pesticide exposures; lack of quantitative information on the timing, duration and extent of exposures; and the absence of appropriate controls for smoking habit, maternal age and previous reproductive history. The following publications were included in a review by Mink et al (2011) of research published over a twelve year period:

- Rull et al (2006) pooled data from two Californian case-control studies evaluating neural tube defects and residential proximity to areas where pesticides were applied; mothers were considered "exposed" if any crop within 1 km had been treated with to glyphosate. Based on 45 exposed cases and 33 exposed controls, ORs of 1.4–1.5 were found depending on the regression model used for analysis. In each instance, the 95% CIs included 1.0.
- In a case-control study performed in an agricultural region of Spain, Garcia et al (1998) observed no significant association between congenital malformations and the fathers' exposure to glyphosate during the three months prior to conception or the first trimester of pregnancy (OR = 0.94; 95% CI = 0.37–2.3).

[6] Fecundability is the probability that conception will occur in a given population of couples during a specific time interval.

- In a population of 2110 Ontario farmers' wives from the Ontario Farm Family Health Study, Arbuckle et al (2001) reported a borderline significant association between pre-conception exposure to glyphosate and spontaneous abortion (33 exposed cases; OR = 1.4; 95% CI = 1.0–2.1), but no significant association with post-conception exposure (22 exposed cases; OR = 1.1; 95% CI = 0.7–1.7). Arbuckle and co-workers considered their investigation as "exploratory" and noted many limitations to their study, including the potential for inaccurate classification of pesticides and timing of exposure relative to conception. They also cautioned that the results should be interpreted with care and confirmed in further investigations.

- To investigate whether reported pesticide use by men or women was associated with delayed pregnancy, Curtis et al (1999) measured the conditional fecundability[7] ratio (CFR)[8] in 2012 planned pregnancies among the Ontario Farm Family Health Study farming couples. The CFR for women who had used glyphosate (regardless of men's use) was depressed (0.61; 95% CI = 0.30–1.3) but there was no statistical significance. Fecundability was slightly elevated (CFR = 1.3; 95% CI = 1.07–1.56) in men who had used glyphosate but whose wives had not. The study authors attributed this finding to uncontrolled factors or chance.

- Self-reported glyphosate exposure during pregnancy was *inversely* associated with gestational diabetes (OR = 0.61; 95% CL = 0.26–1.48) in a cross-sectional analysis of data from the Agricultural Health Study by Suldana et al (2007).

- Self-reported use of glyphosate was associated with a small, statistically non-significant increase in birthweight in the most recent offspring of 700 women in the US Agricultural Health Study (Sathyanarayana et al, 2010).

- Garry et al (2002) conducted a cross-sectional analysis of pesticide applicators and their families. Parent-reported ADD / ADHD in children was associated positively and significantly with use of glyphosate, with 6/14 affected children having parents who had exposure to glyphosate or Roundup (OR = 3.6; 95% CI = 1.35–9.65). ADD / ADHD diagnosis was not confirmed by a clinician, however.

A further review of the scientific literature (Williams et al, 2012) concurred with the conclusion of Mink, i.e., that no consistent effects of glyphosate exposure have been found on reproductive health or offspring development in either humans or animals.

[7] Conditional fecundability is the probability of conception per unit time conditional on a woman being susceptible at the beginning of that time interval.
[8] The ratio of conditional fecundability of the exposed and unexposed groups. A CFR <1.0 indicates a reduced probability of conception in the exposed group.

2.2 The association between glyphosate / glyphosate-based herbicides, endocrine disruption and reproductive toxicity

According to the EOS article:

- Romano et al (2010) have shown that a Roundup formulation was a potent endocrine disruptor in male rats and caused disturbances in reproductive development during puberty. Adverse effects (including delayed puberty and reduced testosterone production) were found at and above the lowest dose of 5 mg/kg.
- Dallegrave (2007) observed adverse reproductive effects in the male offspring of female rats treated with a Roundup formulation at 50, 150 or 450 mg/kg during pregnancy and lactation. The effects, which occurred in the absence of maternotoxicity, included dose-related decreases in serum testosterone level at puberty, decreased sperm number and daily sperm production in adulthood, an increased percentage of abnormal sperm, and sperm cell degeneration.
- Glyphosate active constituent causes sperm damage in rabbits (Yousef et al, 1995).
- When administered to rats for two years at 3, 10 and 32 mg/kg bw/d, glyphosate caused testicular tumours (Lankas, 1981). Although the effect did not occur in a second rat carcinogenicity study at 100, 410 and 1060 mg/kg bw/d, EOS argues that effects related to endocrine hormones can be more potent at low doses than higher ones.

Based on the following evidence, EOS proposes that glyphosate and GBHFs cause reproductive toxicity by mechanisms involving endocrine disruption:

- Glyphosate-based herbicides perturb hormone levels in female catfish and decrease egg viability (Soso et al, 2007) and mediate anti-androgenic and anti-oestrogenic activity in human cells at concentrations as low as 5.0 ppm (Gasnier et al, 2009).
- Roundup reduces production of progesterone in mouse cells *in vitro* by inhibiting expression of a regulatory protein (Walsh et al, 2000).
- Glyphosate disrupts oestrogen-regulated gene expression in human cells (Hokanson et al, 2007) and is toxic to human placental cells, an effect enhanced in the presence of Roundup adjuvants (Richard et al, 2005). Richard et al are said to have shown that Roundup inhibits aromatase (the enzyme responsible for oestrogen production), and proposed this as an explanation for increased premature births and miscarriages reported in female members of farming families using glyphosate (Savitz et al, 1997 and Arbuckle et al, 2001; see previous Section).
- Glyphosate and Roundup damage or kill human umbilical, embryonic and placental cells at concentrations below those recommended for agricultural use, and may interfere with human reproduction and embryonic development (Benachour et al, 2007; Benachour and Seralini, 2009).

APVMA comment

2.2.1 Reproductive effects of glyphosate in vivo

Between them, the German BVL (for the EU and JMPR), Australian DoHA and US EPA have assessed no fewer than eight single- or multi-generation reproduction studies with glyphosate in rats, most of which involved dietary administration. The various agency evaluations are summarised in Appendix 3. The overall dose range was 3 – *ca* 1500 mg/kg bw/d. The toxicological end-points examined included oestrus cycling, mating performance, pregnancy rate and gestation length; litter size and sex ratio; the growth rate, attainment of post-natal developmental landmarks and onset of puberty in pups; and histology of the reproductive organs and analysis of sperm and oocytes in adults. If glyphosate was capable of interfering with the sexual development and reproductive performance of either males or females, the studies would have revealed these effects.

There were few indications of reproductive toxicity. In the parental generations, toxicity was seen as depressed bodyweight or bodyweight gain from doses of *ca* 670 mg/kg bw/d upwards; and, in one study only, histological abnormalities in the salivary glands occurred at $\geq$200 mg/kg. Parental NOELs ranged from 10 to *ca* 700 mg/kg bw/d. Pup bodyweight or bodyweight gain was depressed at $\geq$670 mg/kg, while in one study, litter size was reduced at *ca* 1500 mg/kg bw/d. NOELs in pups varied from 10 to *ca* 800 mg/kg bw/d. The Australian ADI for glyphosate (0.3 mg/kg bw/d) is based on the three-generation dietary study of Schroeder and Hogan (1981), in which there were no treatment-related effects on the parental of filial generations at the highest dose of 30 mg/kg bw/d.

Lower threshold doses for toxicity were seen with glyphosate trimesium in a two-generation study by Stauffer Chemical Co (1983a, assessed by DoHA, 1991). A NOEL of 7.5 mg/kg bw/d was assigned for parental animals and offspring based on reduced bodyweight gain, food consumption and plasma protein levels in adults and depressed pup bodyweight and relative spleen weight at $\geq$40 mg/kg. The only effect on reproductive parameters was a reduction in litter size, which occurred at the highest dose of 100 mg/kg bw/d.

For the EU and JMPR reviews, the BVL also assessed a 13—week US National Toxicology Program study in rats (Chan and Mahler, 1992). Caudal epididymal sperm concentrations declined by *ca* 20% at 25 000 and 50 000 ppm glyphosate in the diet (calculated glyphosate intake *ca* 2500 and 5000 mg/kg bw/d). However, all values were within the HC range and no effects occurred on caudal, epididymal and testicular weights, sperm motility, total spermatid heads/testis and total spermatid heads/gram caudal tissue. Compared with controls, oestrus cycle length was prolonged from 4.9 to 5.4 days at 50 000 ppm. The EU and JMPR regarded this finding as having unknown biological significance, if any. An identical study in male and female mice did not find any evidence of reproductive toxicity or endocrine modulation at up to 50 000 ppm in the diet (7500 mg/kg bw/d), the highest dietary concentration tested.

In an unreliable and poorly-reported study, Yousef et al (1995) administered glyphosate orally to male rabbits for six weeks at 1% or 10% of the LD_{50}. The study authors did not identify the dosing interval, or the doses in terms of mg/kg bw. Semen quality was assessed at weekly intervals for six weeks prior to treatment, during the dosing period, and a further six weeks after treatment to study reversibility of effects.

Glyphosate was claimed to have caused fully or partially reversible decreases in ejaculate volume, sperm viability and sperm activity. However, the results are likely to have been affected by methodological deficiencies, and effects on sperm concentration and morphology are uninterpretable due to major, unexplained variations over time within the control group.

2.2.2 Evidence of endocrine modulation in other studies

Even though they are not specifically designed to test for endocrine disruption, the short-term repeat-dose, subchronic and chronic *in vivo* toxicology studies required by the APVMA and other regulatory agencies can detect modulation of endocrine system activity. Chemicals affecting endocrine target sites initiate direct or compensatory biochemical or cellular responses which are observable by assessment of the weight, gross pathology and histopathology of endocrine organs and tissues. In fact, these studies have some advantages over *in vitro* screening assays, as they assess a variety of endocrine-sensitive endpoints in live animals capable of metabolic activation and/or detoxification of xenobiotic chemicals, and use extended exposure periods encompassing various stages of endocrine development (Williams et al, 2000).

There have been no findings in these subchronic or chronic toxicity studies indicating that glyphosate produces any endocrine-modulating effects. Negative results also were obtained in a dominant lethal mutation study in mice at 2000 mg/kg bw PO (Wrenn, 1980). While this latter test is typically used to assess genetic toxicity, substances that affect male reproductive function through endocrine modulating mechanisms can also produce effects in this type of study (Williams et al, 2000).

2.2.3 Testicular carcinogenicity

A carcinogenicity study by Lankas (1981) has been reviewed by the Australian DoHA (1985), the WHO (1994) and the US EPA (1993). The German BVL did not evaluate this study for the JMPR, but the EU review includes a summary of the WHO assessment. Rats were treated with glyphosate in the diet for 26 months to achieve intakes of *ca* 3, 10 and 31 mg/kg bw/d in males and 3.4, 11 and 34 mg/kg bw/d in females. The incidence of testicular interstitial (Leydig) cell tumours at termination was 0/15 among controls and 2/26, 1/16 and 4/26 at the low-, mid- and high-doses respectively. The total incidence for all males was 0/50, 3/50, 1/50 and 6/50. The BVL evaluator did not attribute the finding to treatment, noting that Leydig cell tumours are common in ageing rats, that the incidence at 31 mg/kg "only slightly exceeded the historical control range," and that no such effect had been observed in several more recent rat studies at much higher doses. In the absence of treatment-related effects, the NOEL was set at 31 mg/kg bw/d.

The WHO (1994), US EPA (1993) and DoHA (1985) all agreed that the tumours were not treatment—related because their incidence lay within the HC range. This interpretation was supported by data shown in the Australian assessment, showing that the incidences of Leydig cell tumours in glyphosate-treated rats were not different to those in male controls from concurrent studies at the same laboratory (4/65, 3/11, 3/26, 3/24 and 3/40).

Furthermore, testicular tumours have not occurred in any of the other carcinogenicity studies with glyphosate in rats or mice at doses of up to 4800 and 1200 mg/kg bw/d, respectively. Despite EOS's claim that endocrine-mediated effects are specifically *low dose* phenomena, doses of between 4 and 12 mg/kg bw/d (within the range given by

Lankas) have failed to cause any testicular effects in two carcinogenicity studies in mice or in three similar studies in rats. Therefore, the weight of evidence does not support EOS's assertion that glyphosate is a testicular carcinogen.

2.2.4 Effects of glyphosate-based herbicide formulations

Notwithstanding the mainly negative findings on glyphosate in carcinogenicity and reproductive toxicity studies in laboratory animals, the APVMA has initiated an independent assessment of publications cited by EOS, and other relevant articles obtained from the scientific literature. Three of these publications describe studies of the effects of GBHFs on the reproductive physiology of rodents and birds, while the remainder cover experiments in isolated cells. The detailed assessments are presented in Appendix 4.

2.2.4.1 Findings in birds

Oliviera et al (2007) observed a 90% reduction in plasma testosterone levels in sexually mature drakes gavaged orally with Roundup (480 g/L glyphosate isopropylamine salt, no other constituents identified) for 15 days at 5 or 100 mg/kg bw/d. This occurred in conjunction with decreased androgen receptor expression within testicular (Sertoli) cells and histological abnormalities in the testis (reduction in seminiferous tubule epithelium and interstitial tissue), epididymal region, proximal efferent ductules (vacuolisation and increased lipid in the epithelium) and epididymal duct (collapsing and folding). As most of these effects were present in birds receiving the lowest dose of 5 mg/kg bw/d, a NOEL was not demonstrated. The study did not investigate whether there were any associated effects on the behaviour or reproductive performance of the birds, define the mechanism by which the effects occurred, or identify the causative component(s) of the test formulation.

2.2.4.2 Findings in rats

Dallegrave et al (2007) performed a single generation reproduction study in rats with a Roundup product (360 g/L glyphosate and 18% POEA surfactant) at maternal oral doses equivalent to 0, 50, 100 and 450 mg glyphosate/kg bw/d. The test formulation was administered to the dams throughout pregnancy and lactation, until the offspring reached 21 days of age. Male pups were then evaluated when 65 or 140 days old. There was no NOEL because of decreased sperm production, an increased incidence of abnormal sperm, and depression in blood testosterone concentration at and above the lowest dose.

In a post-natal development study, Romano et al (2010) treated weanling rats orally with a Roundup product containing 648 g/L glyphosate isopropylamine salt plus unidentified "inert ingredients". The doses were 0, 5, 50 and 250 mg glyphosate/kg bw/d, administered from 23 to 53 days of age. Treated males displayed reduced serum testosterone levels and thinning of the seminiferous tubule germinal epithelium, suggesting diminished production of sperm. Male puberty was delayed at 50 and 250 mg/kg. There was no NOEL.

The APVMA's independent assessment notes that the studies by Dallegrave et al (2007) and Romano et al (2010) appear to have demonstrated evidence of reproductive toxicity. However, both studies are affected by flaws in their design, methodology and / or reporting. Neither research group identified which constituent(s) in the test formulations mediated the reported effects. Also, while there

is a biologically plausible association between delayed puberty, deficiency in circulating testosterone level and inhibited sperm production, the studies did not identify the mechanism involved.

The situation is complicated by a pre / post-natal development experiment by Romano et al (2012), which yielded markedly different findings despite using the same rat strain and Roundup product as did the 2010 study. In the 2012 report, reproductive physiology and behaviour were investigated in male rat pups whose mothers had been dosed orally from GD 18 to PND 5, at 50 mg glyphosate/kg bw/d. The pups were then reared without further exposure until evaluation at 60 days of age. Compared to controls, puberty occurred earlier in the test group; serum testosterone, oestradiol and LH concentrations were doubled; sperm production was enhanced; and males showed a greater preference for the company of female rats despite an increase in the delay before mating. Based on these findings, Romano et al concluded that glyphosate is a potential endocrine disruptor.

However, DeSesso and Williams (2012; see Appendix 4), have questioned several aspects of the study's design and conduct, and observed that the average age and bodyweight of test animals at puberty lay within the range shown by concurrent controls and controls in Romano et al (2010). DeSesso and Williams also note that surfactants likely to be present in the test formulation inhibit steroid production in Leydig (testicular) cells (Levine et al, 2007) and could have affected the study outcome.

2.2.4.3 Findings in vitro

According to the JMPR (2004b), glyphosate had no oestrogenic activity in assays for activation of rainbow trout oestrogen receptors in yeast or vitellogenin production in a trout liver cell culture system (Petit et al, 1997). The incubation concentrations of glyphosate were not given.

A Roundup formulation was reported as having dose-dependently inhibited progesterone synthesis in mouse MA-10 (Leydig tumour) cells (IC_{50} of 24 µg/mL) (Walsh et al, 2000). The putative mechanism involved preventing the expression of steroidogenic acute regulatory (StAR) protein, a mitochondrial phosphoprotein that transfers cholesterol to cytochrome P450scc, the enzyme that initiates steroid hormone biosynthesis. Glyphosate active constituent, by contrast, had no such effect over the concentration range tested (0–100 µg/mL). However, Levine et al (2007) replicated the effect on progesterone synthesis in the same experimental model using 'blank' Roundup formulation (without glyphosate), and demonstrated that inhibition arose from damage to mitochondrial membranes by the surfactant.

In MCF-7 human breast adenocarcinoma (oestrogen sensitive) cells exposed for 18 hours to a GBHF at 0.00023 – 0.23%, significant changes occurred in the activity of three out of 1550 oestrogen-regulated genes. There was a 2.2-fold increase in the activity of HIF1 (which primes cells for the initiation of apoptosis) and *ca* 50% reductions in expression of CXCL12 (a lymphocyte chemoattractant) and EGR1 (which has a range of activities potentially affecting apoptosis and tumour vascularisation) (Hokanson et al, 2007). However, the study did not demonstrate any alteration of the physiology, survival or growth of the test cells, or establish whether the effects on gene expression would have implications for the survival, development and function of other mammalian cells, tissues, foetuses or adult animals.

Furthermore, the formulation component that altered gene expression levels was not identified.

As reported by EOS, a Roundup formulation inhibited aromatase (CYP19, an enzyme which converts androgens to oestrogens) in human plancental cancer (JEG3) cells (Richard et al, 2005; assessed by DoHA, 2005). However, as the DoHA evaluation observed, the use of human placental cancer cells (rather than normal placental cells) was not a valid basis for any conclusion that glyphosate or its products cause reproductive effects in humans, particularly given the weight of evidence from laboratory animals that glyphosate is not a reproductive toxin. Williams et al (2012) have pointed out that the concentrations of Roundup causing aromatase inhibition (0.2–2.0%) in Richard et al's study were cytotoxic and much higher than physiologically relevant; by contrast, pure glyphosate had no effect in the assay system at up to 0.8%, the highest concentration tested. The French Ministry of Agriculture and Fish (2005) has also evaluated Richard et al (2005), and concluded that the study was of no value for human health risk assessment.

Roundup formulations also inhibited aromatase in human embryonic kidney (HEK293) (Benachour et al, 2007) and hepatoma (HepG2) cells (Gasnier et al, 2009). By contrast, glyphosate inhibited aromatase weakly or had no effect on its activity. Roundup formulations had anti-oestrogenic activity at human oestrogen receptors (hER) α or β, and anti-androgenic activity at human androgen receptors (hAR) (Gasnier et al, 2009). However, the potencies of Roundup formulations correlated poorly with the concentration of glyphosate they contained; furthermore, glyphosate itself had no anti-oestrogenic activity at hER α or β and, at most, weak anti-androgenic activity at hAR.

Benachour and Seralini (2009) studied the cytotoxicity of glyphosate, its metabolite AMPA, four Roundup products and the surfactant POEA in three human cell lines (umbilical cord vein endothelial [HUVEC] cells, JEG3 and HEK293). Based on inhibition of mitochondrial respiration, the least potent cytotoxin was AMPA, glyphosate had intermediate potency, and POEA was the most potent (the respective EC50s were $\geq$40 000, *ca* 10 000 and 3–30 ppm). All the product concentrates were more toxic than glyphosate alone, having EC50s of 30 – 9000 ppm. Their potency was not dependent on the concentration of glyphosate they contained, suggesting that other formulation components were biologically active. AMPA and POEA caused necrotic cell death, glyphosate caused cell death via apoptosis, while the Roundup formulations mediated cell death via both necrosis and apoptosis.

Cytotoxicity experiments with isolated rat testicular cells *in vitro* have shown that germ cells are relatively resistant to glyphosate and Roundup Bioforce, Leydig cells are resistant to glyphosate but sensitive to the product at concentrations of $\geq$0.10% in solution, and Sertoli cells are sensitive to glyphosate at $\geq$0.01% and the product at 0.10% (Clair et al, 2012). Notwithstanding the decreases in circulating testosterone levels observed *in vivo*, neither the active nor the formulation inhibited 3β-hydroxysteroid dehydrogenase activity (an index of testosterone synthesis) in cultured Leydig cells exposed for 24 hours at up to 0.10%. Testosterone concentration in the cell incubation medium declined by *ca* 1/3rd in response to glyphosate and Roundup at 0.0001%, but not at higher concentrations. There was no explanation for this paradoxical concentration-response relationship.

2.3 Evidence for the genotoxicity of glyphosate / glyphosate-based herbicides

EOS contradicts the EU review's conclusion that glyphosate is not genotoxic, citing evidence that:

- Roundup increases the frequency of gender-linked recessive lethal mutations in fruit flies (Kale et al, 1995), DNA adducts in the livers and kidneys of mice (Peluso et al, 1998) and sister chromatid exchanges in human lymphocytes (Vigfusson and Vyse, 1980);
- Mice injected with glyphosate and Roundup show an increased frequency of chromosome damage and increased DNA damage in bone marrow, liver and kidney (Bolognesi et al, 1997);
- GBHFs cause DNA damage in human cells (Gasnier et al, 2009);
- In sea urchin embryos, GBHFs and AMPA (the environmental degradation product of glyphosate, aminomethylsulphonic acid) alter cell cycle checkpoints by interfering with DNA repair (Marc et al, 2002; 2004a,b; Belle et al, 2007) and cause inhibition of RNA transcription and delayed hatching (Marc et al, 2005); and
- An epidemiology study in Ecuador found more extensive DNA damage in people living in an area that was aerially sprayed with glyphosate compared with those living 80 km away (Paz-y-Mino et al, 2007).

APVMA comment

The genotoxicity of glyphosate, its metabolite AMPA and GBHFs (with and without surfactants including POEA) has been reviewed by Williams et al (2000), Kier and Kirkland (2013) and the Australian DoHA (1985, 1991, 1992 and 2005), US EPA (1993), WHO (1994) EU (1998) and JMPR (2004b). In addition to assays for gene mutation in bacteria and cultured mammalian cells, the investigated end-points included tests for DNA damage and repair *in vitro* and chromosomal aberrations (clastogenicity) *in vitro* and *in vivo*. All the reviews agreed that the vast majority of studies within the highly extensive database had clearly negative outcomes, and concluded that glyphosate, AMPA and GBHFs do not present a genotoxicity hazard. Furthermore, POEA is not mutagenic (Stegeman and Li, 1990; Williams et al, 2000).

The JMPR and/or EU reviews (both performed by the German BVL) covered four of the studies cited by EOS (2011) as demonstrating genotoxic activity. However, as outlined below, the BVL concluded that the findings were also consistent with cytotoxicity (cellular injury or death not caused by damage to genetic material), and commented that assessment of these data was complicated by a lack of information on product composition, reporting limitations, and by the use of some test systems which were of uncertain relevance for the assessment of risk to humans.

Kale et al (1995) obtained positive results in a test for lethal mutations in fruit flies (*Drosophila melanogaster*) after larvae were treated with a Roundup product (41% glyphosate IPA salt with POEA surfactant) or Pondmaster (41% glyphosate IPA salt with alkyl sulphate surfactant). Dosing conditions were not specified but the test insects were exposed to concentrations close to the LC_{50}. The BVL considered that it

would have been very difficult for the investigators to distinguish between deaths from lethal mutations and deaths from the anticipated high toxicity.

Using a ^{32}P-postlabelling assay, Peluso et al (1998) found a weak, dose-related increase in DNA adducts in the liver and kidney of mice injected IP with 400, 500 and 600 mg/kg of a Roundup product containing 30.4% glyphosate IPA salt with alkyl sulphate surfactant. No adducts were seen with glyphosate IPA alone at 130 or 270 mg/kg, or in a control group. While agreeing that the finding was an indication of possible DNA damage, the BVL regarded the biological significance as equivocal because DNA adducts can occur naturally or arise from increases in endogenous metabolite levels, as well as from direct interaction with chemicals. The BVL also questioned the relevance of IP administration to normal exposure conditions, and criticised the absence of any positive control group, individual animal data and information on the DNA adducts' structure.

Vigfusson and Vyse (1980) observed a weak but statistically significant increase in the frequency of sister chromatid exchanges (SCEs) in human lymphocytes incubated with a Roundup product (composition unspecified) at 250 and 2500 µg/mL. The BVL observed inconsistencies in the results, in that a dose response occurred in cells from only one of the two donors, and the statistically increased values from one donor lay below the control values from the other.

Bolognesi et al (1997) examined the effects of glyphosate and a Roundup product (30.4% glyphosate IPA salt with alkyl sulphate surfactant) on several end-points:

> i) A SCE assay in cultured human lymphocytes from two female donors was positive with glyphosate at 1–6 mg/mL and Roundup at 100 and 330 µg/mL. The formulation was cytotoxic at higher concentrations. The BVL criticised the statistical analysis, as data from the donors were pooled and individual values were not provided.
>
> ii) A weakly positive alkaline elution assay for single-strand DNA breaks and formation of alkali-labile sites in DNA suggested possible transient DNA damage in the liver and kidney of mice, four hours after IP injection with glyphosate or Roundup at 300 and 900 mg/kg respectively. The BVL noted that IP injection was an inappropriate route because the test chemicals could be directly cytotoxic to the tissues within the peritoneal cavity. Furthermore, the outcome was inconsistent with three other studies in which glyphosate did not cause cytogenetic damage, mutation or DNA adduction in mice treated IP at up to 1000 mg/kg bw.
>
> iii) One day after treatment as described in (ii), measurement of 8-hydroxydesoxyguanosine (OHdG) adducts revealed evidence of increased oxidative metabolism / injury in the liver (with glyphosate only) and kidney (with Roundup only). The BVL suggested that the finding may elucidate a mechanism of toxicity but is not evidence of genotoxicity.
>
> iv) In a bone marrow micronucleus assay, groups of three male mice received two IP doses of glyphosate (150 mg/kg) or Roundup (225 mg/kg) at 24—hour intervals, and were killed for assessment six and 24 hours after the final dose. A weakly positive response was obtained with Roundup at both time points, and glyphosate at 24 hours. With respect to glyphosate, the BVL highlighted the inconsistency between the positive outcome and other micronucleus assays, which were negative in rats treated at up to 1000 mg/kg IP and in mice

receiving up to 5000 mg/kg PO. Furthermore, Bolognesi's assay did not comply with the relevant OECD Test Guideline, as the treated groups contained fewer than the recommended five animals and only one dose was tested, precluding the assessment of dose-response. It was unclear when the control mice were killed, weakening the validity of the statistical comparison. The BVL also commented that the formulation (although not the active) may have caused cytotoxicity in the bone marrow, as evidenced by a decrease in the ratio between polychromatic and normochromatic erythrocytes. Cytotoxicity may therefore have affected the frequency of chromosomal aberrations. There was apparently no data on the mutagenicity of the alkyl sulphate surfactant present in the tested Roundup product.

Using the Comet assay, Gasnier et al (2009) measured single- and double-stranded DNA breakage and alkali-labile DNA damage in HepG2 liver cancer cells *in vitro* after 24 hours of incubation with Roundup Grands Travaux, a product containing glyphosate at 400 g/L together with unidentified adjuvants (see assessment in Appendix 3). The test cells were exposed at 1, 2.5, 5, 7.5 and 10 ppm. The pro-mutagen benz[a]pyrene (50 µM) was used as positive control. The test product had no effect at the two lowest concentrations but caused a dose-dependent increase in DNA strand breaks at 5, 7.5 and 10 ppm (50, 60 and 75% breakage compared with 35% in negative controls and 95% in positive controls). However, Gasnier et al also reported that the test product was cytotoxic against HepG2 cells at concentrations of 5 ppm upwards, with an LC50 of 12 ppm. It is therefore possible that the increased DNA strand breakage seen at 5–10 ppm was secondary to cellular injury or death, rather than arising directly from damage to DNA by the test product. Furthermore, it is unclear which component(s) of Roundup Grands Travaux was biologically active, as the effects of glyphosate or adjuvant(s) alone were not tested.

The Australian DoHA (2005) assessment found that Marc et al (2005) had demonstrated that Roundup (diluted to glyphosate concentrations of up to 4 mM) delayed RNA synthesis, transcription of the hatching enzyme and hatching of sea urchin embryos by *ca* two hours. There was only a marginal effect on cell division indicating the delay was not due to any cell-cycle effect. Pure glyphosate at up to 8 mM had only a weak effect on hatching (a delay of 30 min). Marc et al also reported that POEA was "highly toxic to the embryos leading to irreversible damage" but provided no supporting data. The DoHA considered the sea urchin model as being of "dubious" value for human health risk assessment, given that glyphosate had already been tested by validated methods.

In an investigation of associations between genotoxic risk and aerial application of glyphosate-based herbicides for control of illicit crops, Bolognesi et al (2009) performed a cytogenic biomonitoring study on agricultural workers in Colombia. In areas where glyphosate was sprayed, blood samples were taken prior to application and then at five days and four months post-application. Chromosomal damage and cytotoxicity in lymphocytes were evaluated by cytokinesis-block micronucleus assay. Compared with Santa Marta, where organic coffee is grown without pesticides, the baseline frequency of binucleated cells with micronuclei (BNMN) was significantly greater in subjects from four other regions. However, only gender, region and older age were associated with baseline BNMN frequencies, and glyphosate was *not* used in one of the two regions where the highest frequencies of BNMN were found. In three regions, a significant increase in BNMN frequency occurred five days after glyphosate was applied, which reversed in one of these regions within four months

post-application. The study authors concluded that genotoxic damage associated with glyphosate application was small and transient, and the genotoxic risk was low.

2.4 Carcinogenicity of glyphosate / glyphosate-based herbicides

2.4.1 Evidence from studies in laboratory animals

The EOS article claims that glyphosate is carcinogenic, based on an increase in testicular tumours in rats treated via their diet for two years at 3, 10 and 32 mg/kg bw/d. However, pesticide regulatory agencies have not classified glyphosate as a carcinogen because the effect did not occur at higher doses in another two-year rat study. EOS argues that endocrine effects are more potent at low doses than higher doses, and so regulators should re-classify glyphosate as a carcinogen. EOS also claims that George et al (2010) have demonstrated that glyphosate induces cancer in mouse skin.

APVMA comment

2.4.1.1 Carcinogenicity via the oral route

The study in which testicular tumours occurred (Lankas, 1981) has been reviewed by the Australian DoHA (1985), WHO (1994) and US EPA (1993). The German BVL did not evaluate this study for the JMPR (2004b), but the EU review includes a summary of the WHO assessment. Rats were treated with glyphosate for 26 months at dietary doses of *ca* 3, 10 and 31 mg/kg bw/d in males and 3, 11 or 34 mg/kg bw/d in females. The incidence of testicular interstitial (Leydig) cell tumours at termination was 0/15 among controls and 2/26, 1/16 and 4/26 at the three respective doses. The total incidence for all males was 0/50, 3/50, 1/50 and 6/50. The BVL did not attribute the finding to treatment, noting that Leydig cell tumours are common in ageing rats, that the incidence at 31 mg/kg "only slightly exceeded the historical control range," and that no such effect had been observed in several more recent rat studies at much higher doses. In the absence of treatment-related effects, the NOEL was set at 31 mg/kg bw/d.

The WHO (1994), US EPA (1993) and DoHA (1985) all agreed that the tumours were not treatment-related because their incidence lay within the HC range. This interpretation was supported by data shown in the Australian assessment, showing that the incidences of Leydig cell tumours in glyphosate-treated rats were not different to those in male controls from concurrent studies at the same laboratory (4/65, 3/11, 3/26, 3/24 and 3/40).

Furthermore, glyphosate has not caused cancer in the testis – or at other sites – in any of the other dietary carcinogenicity studies assessed the Australian DoHA (1985, 1991 and 1992), US EPA (1993), EU (1998) and JMPR (2004b). The database comprises:

- A 20-month study in mice at *ca* 11.3 – 45 mg/kg bw/d (Indian Institute of Toxicology, undated);
- A 22-month study with glyphosate trimesium in male and female mice treated at 11.7 – 991 and 16.0 – 1341 mg/kg bw/d respectively (Stauffer Chemical Co, 1987a);
- Two-year studies in mice at 100 – 1000 mg/kg bw/d (Atkinson et al, 1993a) and 157 – 4841 and 190 – 5874 mg/kg bw/d in males and females, respectively (Knezevich and Hogan, 1983); and

- Two-year studies in rats at 89 – 940 and 113 – 1183 mg/kg bw/d in males and females respectively (Stout and Ruecker, 1990); 10 – 1000 mg/kg bw/d (Atkinson et al, 1993b); 121 – 1214 and 145 – 1498 mg/kg bw/d in males and females (Brammer, 2001); 6.3 – 595 and 8.6 – 886 mg/kg bw/d in males and females (Suresh, 1996); and at 4.2 – 41.8 and 5.4 – 55.7 mg/kg bw/d in males and females (glyphosate trimesium salt; Stauffer Chemical Co, 1984).

Despite EOS's argument that endocrine-mediated effects are specifically *low dose* phenomena, dietary doses of between 4 and 16 mg/kg bw/d (which lie within the range given by Lankas, 1981) have failed to cause any testicular effects in two mouse and three rat carcinogenicity studies. Therefore, the weight of evidence does not support the EOS assertion that glyphosate is a testicular carcinogen.

2.4.1.2 Dermal carcinogenicity

George et al (2010) tested Roundup Original (a product containing 360 g/L glyphosate and 15% POEA) in a mouse two-stage initiation / promotion model of skin cancer. Following a single dermal dose of the tumour initiator DMBA (7,12-dimethyl benz[a]anthracene) mice were treated dermally, three times per week for 32 weeks, with Roundup (25 mg/kg bw) or a positive control chemical (the tumour promoter TPA (12-*O*-tetradecanoyl-phorbol 13-acetate) at 5 µg/mouse). Skin cancers (squamous cell papillomas) were present on eight/20 Roundup-treated mice and 20/20 positive controls at termination. By contrast, tumours did not develop on untreated (negative control) animals or further mice that received a single dose of DMBA without a promoter; or 32 weeks' treatment with Roundup or TPA without prior initiation; or one dose of Roundup followed by TPA for 32 weeks.

Before discussing the significance of George et al's findings, we must briefly consider the biological basis for the two-stage initiation / promotion model they utilised. This experimental model has been developed in light of the *multistage model of carcinogenesis*[9], the current scientific explanation of how cancers are formed from normal cells. In their experimental design, George et al used a single dose of DMBA to initiate skin tumours and repeated doses of TPA to promote them. Tumours did not develop on animals that received the initiator without subsequent promotion, or on mice treated with the promoter without prior initiation. When substituted for DMBA, Roundup did not behave as a tumour initiator, as tumours did not form on mice treated subsequently with TPA. Furthermore, Roundup was not a complete carcinogen, since tumours did not develop on animals that received it without prior initiation. However, Roundup did behave as a tumour promoter on mice that had already received DMBA.

[9] As described by Derelanko (2002), the development of a single cell into malignant tumours is believed to occur in three stages, the first of which is **initiation** (a normal cell changes irreversibly – usually by genetic alteration – in a way that allows unrestricted division; however, initiated cells may remain latent for months or years, during which they are indistinguishable from normal). The subsequent stage, **promotion**, involves prolonged and repeated exposure to a promoting agent which causes the initiated cell to undergo clonal expansion and form a pre-cancerous focus. Promoters, which do not interact directly with DNA, are believed to act via a variety of mechanisms most often resulting in increased cellular replication. The final step is **progression**, in which the pre-cancerous focus becomes transformed into a malignant tumour, a process characterised by changes in the number and arrangement of chromosomes, an increased rate of replication, and invasiveness.

Because George et al did not apply pure glyphosate or POEA to the test animals, their study could not identify which component(s) of Roundup Original was responsible for the promoting activity. Therefore, EOS's assertion that "glyphosate induces cancer in mouse skin" is not strictly correct. Furthermore, while single doses of Roundup and TPA induced similar changes in dermal protein expression, it remains unclear whether the formulation and positive control shared a common mode of action (see assessment in Appendix 3).

However, the most important issue raised by this study is whether Roundup Original or other GBHFs are likely to pose a dermal carcinogenicity hazard to persons preparing them for application. In this regard, several factors require consideration:

- The weight of evidence suggests that neither glyphosate nor POEA are genotoxins, either alone or in combination. Furthermore, glyphosate has been shown not to be carcinogenic via the oral route in ten studies in two laboratory species.
- Roundup Original was not a complete carcinogen in the mouse initiation / promotion model. Tumour initiation was a prerequisite for the eventual development of dermal cancers. Therefore, this and similar products would not be expected to promote tumour formation on human skin in the absence of prior initiation.
- Roundup Original was a markedly less potent promoter than the positive control, TPA. George et al applied the formulation at a 150-fold higher dose than TPA (25 mg/kg bw compared with 5 µg/mouse, equivalent to *ca* 0.17 mg/kg assuming a 30 g bodyweight). Despite this, Roundup promoted tumour formation more slowly than did the positive control. Tumours first appeared after 130 days on Roundup-treated mice, compared with 52 days on those receiving TPA. Fewer, smaller tumours developed on Roundup-treated mice than on those receiving TPA. Moreover, tumour formation occurred on all positive control mice, compared with 40% of those receiving Roundup.
- Tumour promotion is reversible, requires prolonged and repeated exposure to the promoter, and the promoted cell population depends on the continued presence of the promoter (Derelanko, 2002). On mice, tumours did not appear until 130 days of treatment with Roundup Original. Assuming a lifespan of 80 years, humans would have to be exposed to Roundup for three days per week for *ca* 14 years to achieve the equivalent of 130 days of the *ca* 730-day mouse lifespan. Few herbicide mixer / loaders, if any, would experience such prolonged uninterrupted exposure, especially in situations where GBHFs have a seasonal pattern of use.
- Mice received Roundup Original at 25 mg/kg bw/d, which is equivalent to 1500 mg/d for a 60 kg human. The mass of Roundup formulation that must be handled per day to attain a dermal dose of 1500 mg can be estimated using the US EPA (2012) Exposure Surrogate Reference Table. Based on monitoring studies of operators mixing and loading liquid pesticide concentrates under field conditions, this nominates a mean unit dermal exposure of 0.083 mg/kg handled for persons wearing a single clothing layer and gloves[10]. Therefore, to

[10] Label Safety Directions for liquid glyphosate-based professional strength products require users to wear PPE including coveralls and gloves, consistent with recommendations in the Handbook of First Aid Instructions and Safety Directions (DoHA, 2012).

attain a dermal exposure of 1500 mg, 1500 ÷ 0.083 = 18 072 kg of the product would have to be handled, which is at least ten times higher than could be achieved in a working day.

2.4.2 Evidence from human populations

Citing human epidemiology studies by De Roos et al (2005), Hardell and Eriksson (1999), Hardell et al (2002) and Eriksson et al (2008), EOS claims that there is an association between exposure to glyphosate / GBHFs and the blood system cancers multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL).

APVMA comment

In 2005, the Australian DoHA evaluated epidemiological evidence of associations between use of glyphosate and cancer.

- According to the DoHA, McDuffie et al (2001) found no significant association between previous use of Roundup and the occurrence of Non-Hodgkin's Lymphoma (NHL) among Canadian men (119 test and 301 control), although the study did suggest an association between increased risk of NHL and the use of multiple pesticides.
- The Agricultural Health Survey, a prospective cohort study of 57 311 licensed pesticide applicators in Iowa and North Carolina (De Roos et al, 2005a) found no association between glyphosate exposure and NHL. Based on 22 of 32 cases[11], mixing or using glyphosate products was claimed to be associated with an elevated risk of multiple myeloma (MM), with an odds ratio of 2.6 (95% Confidence Interval = 0.7–9.4), although the lower CI of 0.7 limited the strength of the finding. There was also a possible relationship between the risk of MM and the cumulative exposure days (years of glyphosate use X days per year) but not intensity-weighted exposure (years of glyphosate use X days X intensity level). However, when Sorahan (2012) re-analysed the complete dataset of 32 cases, the relative risk for ever using glyphosate was only 1.1 (95% CI = 0.5–2.4) when adjusted for age. Additional adjustment for education, smoking, alcohol use, family history of cancer and use of 10 other pesticides had little effect (OR = 1.2; 95% CI = 0.5–2.9). This demonstrates that glyphosate use is not associated with increased risk of MM.
- De Roos et al (2005b) found a possible association between NHL and the use of glyphosate in a pooled analysis of 650 males participating in case-control studies performed by the US National Cancer Institute during the 1980s. An OR of 2.1 (95% CI = 1.1–4.0) was detected by logistic regression, but the association was weaker (OR = 1.6; 95% CI = 0.9–2.8) when analysed by hierarchical regression.
- In a study of 515 cases and 1141 controls, Hardell et al (2002) obtained elevated risk of NHL or hairy cell leukaemia (HCL) among men who had used glyphosate. However, the DoHA considered the finding as equivocal because of the small sample size (8 cases and 8 controls), inconsistency between the odds ratios obtained by univariate analysis (3.04; 95% Confidence Interval =

[11] De Roos et al reduced the dataset from 32 to 22 MM cases by excluding subjects with missing data for several variables (Sorahan, 2012).

1.08–8.52) and multivariate analysis (1.85; 95% CI = 0.55–6.20), and the wide breadth of the 95% confidence intervals.

In a follow-up study (see assessment in Appendix 3), Eriksson et al (2008) examined exposure to pesticides as a risk factor for NHL in 910 cases and 1016 controls. Univariate analysis revealed a significant association between NHL and exposure to glyphosate (29 cases and 18 controls; OR = 2.02; 95% CI = 1.10–3.71), exposure to glyphosate with a latency of >10 years between exposure and diagnosis (OR = 2.26; 95% CI = 1.16 – 4.40) and exposure to glyphosate for >10 days (17 cases and 9 controls; OR = 2.36; 95% CI = 1.04–5.37). However, NHL was not associated with exposure to glyphosate with a latency of 1–10 years between exposure and diagnosis (OR = 1.11; 95% CI = 0.24 – 5.08) and was, at most, only weakly associated with exposure to glyphosate for <10 days (12 cases and 9 controls; OR = 1.69; 95% CI = 0.70 – 4.07). Multivariate analysis did not demonstrate any association between NHL and glyphosate exposure (OR = 1.51; 95% CI = 0.77–2.94).

Of the epidemiology studies assessed in Australia, three have suggested an association between glyphosate use or exposure and NHL, but obtained inconsistent results depending on the type of statistical analysis performed. Two other studies have searched for but did not find any such association. Possible associations between glyphosate and HCL and MM were observed in one study each, although the association with MM has subsequently been discounted following a re-analysis of the data.

When weighing up the significance of these results, it is worth taking account of the limitations in the design of the studies, which (with the exception of De Roos, 2005a) collected exposure data in questionnaires relying on the accuracy of the respondent's memory. This would result in recall bias, misclassification of pesticide exposure, and increased uncertainty regarding the actual level of exposure. Epidemiological studies of this type are also potentially confounded by exposure to multiple pesticides and by established risk factors for haematopoietic system cancers, such as immunosuppression and Epstein-Barr virus (DoHA, 2005).

The JMPR (2004b) review of glyphosate reached similar conclusions from its assessment of epidemiology studies by Hardell and Eriksson (1999), Nordstrom et al (1998) and McDuffie et al (2001), commenting that the claimed associations between glyphosate and lymphopoietic cancers were weak, were not controlled for confounding factors including other pesticides, and did not meet generally accepted criteria for determining causal relationships.

2.5 Neurotoxicity of glyphosate / glyphosate-based herbicides

The EOS article describes glyphosate as an organophosphate, and asserts that it has shown a range of neurotoxic effects. These include neurobehavioural disorders in the children of pesticide applicators (Garry et al, 2002), Parkinson's disease in a man who accidently sprayed himself (Barbosa et al, 2001), biochemical abnormalities in rat brain cells including depletion of the neurotransmitters serotonin and dopamine (Anadon et al, 2008) and loss of mitochondrial trans-membrane potential (Astiz et al, 2009), and synergistic toxicity with diazinon towards neuroblastoma (nerve cancer) cells *in vitro* (Axelrad et al, 2003).

APVMA comment

Glyphosate is an organic chemical containing a phosphorus atom, but does not exhibit the same biological activity as organophosphate insecticides. In fact, there is a substantial body of evidence from laboratory animal studies that glyphosate does not affect cholinesterase (ChE) activity in the brain or blood, or cause acute, delayed or chronic toxicity to the nervous system.

In an acute neurotoxicity study with glyphosate trimesium in rats gavaged at 645, 968 and 1290 mg/kg bw, the mid and high doses caused behavioural depression, hypothermia and deaths but no inhibition of brain or RBC ChE activity. Glyphosate trimesium did not depress ChE activity in a two-year dietary study in rats at up to 42 (males) / 56 (females) mg/kg bw/d (Stauffer Chemical Co, 1984), in a two-generation rat reproduction study at dietary doses up to *ca* 100 mg/kg bw/d (Stauffer Chemical Company, 1983) or in dogs gavaged at up to 50 mg/kg bw/d for 12 months (Stauffer Chemical Co, 1987b) (DoHA, 1991).

The JMPR (2004b) review of glyphosate included BVL evaluations of acute (single oral dose) and 13–week (dietary administration) neurotoxicity studies in rats, performed according to OECD Test Guideline 424 (Horner, 1996a,b). Despite the occurrence of general toxicity, there was no behavioural or histological evidence of toxicity to the central or peripheral nervous systems at the respective highest doses of 2000 mg/kg bw and 1547 mg/kg bw/d. Similarly, glyphosate displayed no acute delayed neurotoxicity when tested in chickens by OECD Test Guideline 418 at an oral dose of 2000 mg/kg bw (Johnson, 1996). There was no treatment-related depression in brain acetylcholinesterase (AChE) activity or neuropathy target esterase activity in the brain or spinal cord.

The EU review of glyphosate included BVL assessments of two 21-day oral repeat-dose neurotoxicity studies in chickens, performed with glyphosate at up to 1000 mg/kg bw/d (Bhide, 1987) and Glycel 41 SL at doses up to an equivalent of 1600 mg glyphosate/kg bw/d (Bhide, 1988d). Both studies investigated behaviour, spinal cord and sciatic nerve histology, plasma ChE activity, haematology and clinical chemistry. Slight ataxia (loss of touch sensation) occurred in 1/3 high dose hens on day 18 of Bhide (1987), but otherwise there was no behavioural or histological evidence of neurotoxicity, and no depression in ChE activity. The EU concluded there was no primary neurotoxic effect. The Australian DoHA (1992) assessment of Bhide (1987) agreed that there was no neurotoxicity or neurological change in the spinal cord or peripheral nerves.

The case report of Parkinson's disease in a man following exposure to glyphosate (Barbosa et al, 2001) is inconsistent with previous findings in animals and humans,

and insufficient to prove a causal relationship (JMPR, 2004b). In a review of published epidemiological studies, Mink et al (2011) cite a case-control study (Weschler et al, 1991) reporting an unadjusted OR of 4.04 for Parkinson's disease and use of Roundup at home, based on 19 cases (14 exposed) and 22 controls (9 exposed). However, the strength of the association is questionable due to the small sample size and variability in the data (the 95% CI of 0.91–19.3 was very wide and included 1.0). Furthermore, there was no association between glyphosate exposure and Parkinson's disease in a much larger cohort study of pesticide applicators and their spouses (Kamel et al, 2007), either at enrolment (relative risk of 1.1 in 79 640 subjects) or follow—up (RR of 1.0 in 56 009 subjects).

Garry et al (2002) conducted a cross-sectional analysis of pesticide applicators and their families. Parent-reported ADD / ADHD in children was associated positively and significantly with use of glyphosate, with 6/14 affected children having parents who had exposure to glyphosate or Roundup (OR = 3.6; 95% CI = 1.35–9.65). ADD / ADHD diagnosis was not confirmed by a clinician, however (Mink et al, 2011). The biological significance of findings by Anadon et al (2008), Astiz et al (2009) and Axelrad et al (2003) is unknown, and it is uncertain whether these studies are indicative of any hazard to humans.

The APVMA will monitor the scientific literature for future developments in this area, ensure that relevant research reports are reviewed, and take action if required.

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APPENDIX 1: ASSESSMENTS OF DEVELOPMENTAL STUDIES IN RATS

Summary Table: Developmental toxicity studies in rats – Percentage incidences of foetal anomalies and malformations

Reference	Assessor	Treatment-related Findings	Dose (mg/kg bw/d) Green = foetal NOEL Red = foetal LOEL			
Brooker et al (1991a)	**BVL for JMPR & EU**		**0**	**300**	**1000**	**3500**
		Wavy ribs	CC: 0.6	0	1.8	19.7
		Reduced ossification of cranial centre(s)	CC: 1.9	1.4	7.2	6.9
		Reduced ossification of sacro-caudal vertebral arches	CC: 1.9	5.6	10.2	10.4
		Unossified sternebrae	CC: 13.7	28.5	17.6	33.8**
		Skeletal anomalies (all)	CC: 11.7 HC: 21.9-27.2	22.6	28.4*	35.7**
Tasker et al (1980a)	**BVL for EU**		**0**	**300**	**1000**	**3500**
		Foetal malformations (all)	CC: ? HC: ?	?	?	?
		Unossified sternebrae	CC: ?	?	?	?
	Australia DoHA		**0**	**300**	**1000**	**3500**
		Unossified sternebrae	CC: ?	?	?	?
	US EPA		**0**	**300**	**1000**	**3500**
		Unossified sternebrae	CC: ?	?	?	?
Moxon (1996a)	**BVL for JMPR**		**0**	**250**	**500**	**1000**
		None	NR	NR	NR	NR
Suresh (1991)	**BVL for EU**		**0**		**1000**	
		None	NR		NR	
Bhide (1986)	**BVL for EU**		**0**	**100**	**500**	
		None	NR	NR	NR	
	Australia DoHA		**0**	**100**	**500**	
		None	NR	NR	NR	
Anon (1981)^	**BVL for EU**		**0**	**22**	**103**	**544/558**
		None	NR	NR	NR	NR
Stauffer Chemical Co (1982)^^	**Australia DoHA**		**0**	**30**	**100**	**333**
		None	NR	NR	NR	NR

Statistical significance vs concurrent control group: *p < 0.05 **p<0.01
CC = Concurrent control group mean HC = Historical control group range NR = None reported
? = No incidence data provided in assessment. ^Glyphosate administered in the diet; otherwise gavage dosing.
^^Glyphosate trimesium

Brooker et al (1991a) [Reviewing Agency: BVL] The BVL assessed this study for both the JMPR (2004b) and EU (1998) reviews of glyphosate. There were no discrepancies between the two evaluations, although the EU report provided more data on skeletal ossification. In rats orally gavaged from GD 6 – 15 at 0, 300, 1000 or 3500 mg/kg bw/d, there were maternal deaths at 3500 mg/kg and other evidence of maternotoxicity (clinical signs and a dose-related reduction in bodyweight gain) at 1000 and 3500 mg/kg. Litter and mean foetal weights were depressed at 3500 mg/kg. The incidence of malformations was not affected by treatment but at 1000 and/or 3500 mg/kg, there were increased incidences of wavy ribs and deficits in ossification of the cranium, vertebral arches and sternebrae (see Table). The proportion of foetuses

displaying skeletal anomalies was elevated significantly at 1000 and 3500 mg/kg compared with concurrent controls. The incidence of skeletal anomalies was also increased at 300 mg/kg, but lay within the HC range (the BVL also noted that the study control incidence of skeletal variations was atypically low). The finding in this particular group was therefore considered not to be treatment-related, so the NOAEL for maternotoxicity and developmental toxicity was set at 300 mg/kg bw/d. Kimmel et al (2013) set maternal and foetal NOAELs of 1000 mg/kg bw/d in an evaluation of this study, without commenting on the skeletal anomalies.

Tasker et al (1980a) [Reviewing Agencies: BVL, US EPA and Australian DoHA] According to the BVL evaluation for the EU, glyphosate was administered by gavage to rats over GD 6 – 19 at 0, 300, 1000 or 3500 mg/kg bw/d. Maternal toxicity (mortality, clinical signs and reduced bodyweight gain), enhanced foetal mortality, depressed foetal bodyweight and a higher incidence of unossified sternebrae occurred at 3500 mg/kg. At this dose there was also an increased number of foetuses with malformations (which the BVL did not describe). However, since the incidence and type of malformations were similar to those from HC data, the BVL did not ascribe them to treatment. No further information was provided. NOELs of 1000 mg/kg bw/d were therefore set for maternal and foetal toxicity.

The US EPA (1993) assessment of Tasker et al (1980a) agreed with the BVL evaluation. In the presence of maternotoxicity at 3500 mg/kg bw/d, foetal developmental effects were assessed as increased numbers of foetuses and litters with unossified sternebrae, and decreased mean foetal bodyweight. The NOAEL for maternal toxicity and developmental toxicity was set at 1000 mg/kg bw/d.

The Australian DoHA (1985) evaluated Tasker et al (1980a) in greater detail than the other two agencies but agreed with their principal findings. At 3500 mg/kg, signs of maternal toxicity comprised decreased bodyweight gain; diarrhoea, soft stools, reduced activity and rales in all dams from half way through the dosing period, and six maternal deaths. Dams receiving 300 and 1000 mg/kg showed no reaction to treatment. The NOEL for maternotoxicity was therefore set at 1000 mg/kg bw/d. Increases in the number of foetuses with unossified sternebrae (a developmental variation), dwarfism and bent tail were noted at 3500 mg/kg. However, all dwarf foetuses were in one litter, all foetuses with bent tail were from another litter, and the control and 3500 mg/kg groups had the same number of litters containing malformed foetuses. HC data indicated there were five bent tails out of 5008 foetuses, all confined to one litter out of 383. The DoHA therefore attributed dwarfism and bent tail to genetic factors and in the absence of foetal malformations at 1000 or 300 mg/kg, set a NOEL of 1000 mg/kg bw/d fetotoxicity. Assessments of this study by Williams et al (2012) and Kimmel et al (2013) made the same conclusions.

Moxon (1996a) [Reviewing Agency: BVL] This study, in which dams were orally gavaged from GD 7 – 16 at 0, 250, 500 or 1000 mg/kg bw/d, was assessed for the JMPR review only. There were no treatment-related findings, and so NOELs of 1000 mg/kg bw/d were set for maternal and developmental toxicity. The BVL's conclusions have been corroborated independently by Kimmel et al (2013).

Suresh (1991) [Reviewing Agency: BVL] Rats received glyphosate at 0 or 1000 mg/kg bw/d by gavage between GD 6 and 15. There was no evidence of maternotoxicity, embryolethality or foetal malformations in the treated group, but

there was a higher incidence of delayed ossification of the caudal vertebral arch and proximal forelimb and distal hindlimb phalanges. However, delayed ossification of other parts of the skeleton, particularly the skull, was more frequently seen in the control group. As there was "no clear and consistent impact of test compound administration" on ossification, NOELs of 1000 mg/kg bw/d were set for maternal and developmental toxicity. Kimmel et al (2013) also accepted there were no treatment-related effects in this study.

Bhide (1988a) [Reviewing Agencies: BVL and Australian DoHA] The BVL and DoHA (1992) assessments of this study were closely similar. Glyphosate was administered to rats from GD 15 to LD 21 at nominal doses of 0, 50 and 100 mg/kg bw/d. The bodyweight and food consumption of dams were unaffected, and there were no treatment-related effects on litter parameters including pup bodyweight, survival or growth. No pathological examination was performed. Both agencies set the NOEL in parents and offspring at 100 mg/kg bw/d.

Bhide (1986) [Reviewing Agencies: BVL and Australian DoHA] Again, the two agencies' evaluations coincided. No treatment-related maternal or foetal effects were observed in rats gavaged with glyphosate at 0, 100 or 500 mg/kg bw/d on GD 6 to 15. NOELs for materno- and fetotoxicity were set at 500 mg/kg bw/d. The EU review classified this study as "supplementary" due to reporting deficiencies.

Anon (1981) [Reviewing Agency: BVL] Glyphosate was administered in the diet to rats over GD 6 – 18. Achieved doses were 0, 22, 103 and 544 mg/kg bw/d. There was no materno- or fetotoxicity, and no foetal malformations were recorded. At the highest dietary concentration, an additional group of rats was allowed to litter and nurse their pups until LD 28. Their achieved dose was 558 mg/kg bw/d. No treatment-related effects were observed either in the dams or pups. The EU classified this study as "supplementary" due to reporting deficiencies.

Stauffer Chemical Company (1982) [Reviewing Agency: Australian DoHA] Glyphosate trimesium was administered by gavage to pregnant rats over GD 6 – 20 at 0, 30, 100 or 333 mg/kg bw/d. At the high dose, there was maternotoxicity seen as mortality, clinical signs, reduced bodyweight gain and food consumption. The maternal NOEL was therefore 100 mg/kg bw/d. No treatment-related effects on foetal survival or development occurred, but mean foetal bodyweight was depressed at 333 mg/kg bw/d. A NOEL of 100 mg/kg bw/d was set for fetotoxicity (DoHA, 1991).

Studies with aminomethylphosphonic acid (AMPA)

In addition to developmental toxicity studies on the parent chemical, the EU review included BVL assessments of oral gavage studies in pregnant rats with the glyphosate metabolite AMPA. Following a range finding experiment which found no maternal or foetal effects at up to and including the highest dose of 1000 mg/kg bw/d **(Holson, 1991a)**, AMPA was given at 0, 150, 400 or 1000 mg/kg bw/d from GD 6 through 15 **(Holson, 1991b)**. Dams displayed hair loss and mucoid faeces at 400 and 1000 mg/kg together with transient depression in bodyweight gain and food consumption at 1000 mg/kg only. Foetal bodyweight was slightly but significantly reduced at 1000 mg/kg, but there was no evidence of developmental malformations. Accordingly, the BVL set NOELs of 150 and 400 mg/kg bw/d for maternal and foetal toxicity. Williams et al

(2012) have confirmed these findings, although reporting the maternal NOEL as 400 mg/kg bw/d. No treatment-related maternal or foetal effects occurred when AMPA was administered to pregnant rats at 0, 100, 350 or 1000 mg/kg bw/d over GD 6 – 16 **(Hazelden, 1992)**.

APPENDIX 2: ASSESSMENTS OF DEVELOPMENTAL STUDIES IN RABBITS

Developmental toxicity studies in rabbits: Incidences of foetal mortality, anomalies and malformations

Reference	Assessor	Treatment-related Findings	Dose (mg/kg bw/d) Green = foetal NOEL Red = foetal LOEL			
Brooker et al (1991b)	**BVL for JMPR & EU**		**0**	**50**	**150**	**450**
		Late embryonic deaths (Mean no./litter)	CC: 0.2 HC: 0.1-1.3	0.9	0.5	1.3**
		Postimplantation loss (%)	CC: 5.7 HC: 6.5-17.5	19.5*	15.3*	21.0**
		Malformations (all) (%)	CC: 1.8	2.9	4.5	6.3
		Intraventricular septal defect & other cardiac abnormalities (%)	CC: 0.6 HC: 0.7-5.9	1.0	3.6	5.3
	Kimmel et al (2013)		**0**	**50**	**150**	**450**
		Embryofetal deaths (Mean no./litter)	CC: 0.6	1.8*	1.5*	1.8**
		Postimplantation loss (%)	CC: 5.7	19.5*	15.3*	21.0**
		Malformations (all) (%)	CC: 1.8	2.9	4.5	6.3
		Intraventricular septal defect & other cardiac abnormalities (%)	CC: 0.6	1.0	3.6	5.3
Bhide and Patil (1989)	**BVL for EU**		**0**	**125**	**250**	**500**
		Viable implants (Mean no./litter)	CC: 7.3	8.0	8.0	5.2
		Non-viable implants (Mean no./litter)	CC: 0.07	0.13	0.27	1.4
		Ventricular septal defect (%)	CC: 0	0.9	0.8	2.6
		Postcaval lung lobe absent (%)	CC: 0	0.9	1.6	5.1
		Kidney(s) absent (%)	CC: 0.9	1.8	1.6	7.7
		Rudimentary 14th rib, unilateral (%)	CC: 0.9	0	1.6	6.4
	Kimmel et al (2013)		**0**	**125**	**250**	**500**
		Embryofetal deaths (Mean no./litter)	CC: 0.07	0.13	0.27	1.4
		Total no. foetuses with visceral malformations	CC: 1	4	5	12
		Total no. foetuses with cardiovascular malformations	CC: 0	1	1	2
		Total no. foetuses with skeletal malformations	CC: 1	0	2	5
Moxon (1996b)	**BVL for JMPR**		**0**	**100**	**175**	**300**
		Partially ossified transverse process, 7th vertebra (%)	CC: 0.7	NR	NR	5.6
		Unossified transverse process, 7th lumbar vertebra (%)	CC: 2.8	NR	NR	9.7
		Partially ossified 6th	CC: 2.8	NR	NR	11.1

Reference	Assessor	Treatment-related Findings	Dose (mg/kg bw/d) Green = foetal NOEL Red = foetal LOEL			
		sternebra (%)				
	Kimmel et al (2013)		**0**	**100**	**175**	**300**
		Postimplantation loss (%)	CC: 11.7	9.5	12.1	13.6
		Total no. foetuses with cardiovascular malformations	CC: 1	1	0	1
		Total no. foetuses with major skeletal malformations	CC: 3	0	0	1
		Total no. foetuses with minor skeletal malformations	CC: 58	82**	59	79**
		Total no. foetuses with skeletal variations	CC: 119	129	116	132**
Suresh (1993a)	**BVL for EU**		**0**	**20**	**100**	**500**
		Dilated heart (%)	CC: 0	5.1*	5.2*	17.9*
		Major visceral malformations (all) (%)	CC: 3.0	7.7	7.7	29.6
		Extra 13th rib (%)	CC: 0	1.3	2.6	3.6*
	Kimmel et al (2013)		**0**	**20**	**100**	**500**
		Embryofetal deaths (Mean no./litter)	CC: 0.90	1.38	2.00	1.67
		Postimplantation loss (%)	CC: 13.5	18.6	23.4	23.2
		Total no. foetuses with visceral malformations	CC: 4	6	6	8*
		Total no. foetuses with cardiovascular malformations	CC: 2	4	6	6
		Total no. foetuses with “seal-shaped” heart	CC: 1	0	0	0
		Total no. foetuses with “seal-shaped” heart & cardiomegaly	CC: 0	0	1	0
		Total no. foetuses with dilated heart	CC: 0	4*	4*	5*
		Total no. foetuses with dilated ventricle	CC: 1	0	1	1
		Total no. foetuses with skeletal malformations	CC: 11	5	0	1
Tasker et al (1980b)	**BVL for EU**		**0**	**75**	**175**	**350**
		None	NR	NR	NR	NR
	Australia DoHA		**0**	**75**	**175**	**350**
		None	NR	NR	NR	NR
	US EPA		**0**	**75**	**175**	**350**
		None	NR	NR	NR	NR
	Kimmel et al (2013)		**0**	**75**	**175**	**350**
		Postimplantation loss (%)	CC: 16.7	4.9	2.5	18.7
		Total no. foetuses with cardiovascular malformations	CC: 0	0	0	0
		Total no. foetuses with skeletal malformations	CC: 0	3	2	0
Anon (1981)^	**BVL for EU**		**0**	**10.5**	**50.7**	**255**
		Foetal loss (%)	0.9	0.8	6.1	7.0
Stauffer	**Australia**		**0**	**10**	**40**	**100**

Reference	Assessor	Treatment-related Findings	Dose (mg/kg bw/d) Green = foetal NOEL Red = foetal LOEL			
Chemical Co (1983b)^^	**DoHA**	None	NR	NR	NR	NR
Coles and Doleman (1996)	**Kimmel et al (2013)**		**0**	**50**	**200**	**400**
		Embryofetal deaths (Mean no./litter)	CC: 0.36	0.33	1.00*	1.40
		Postimplantation loss (%)	CC: 3.7	3.6	11.5*	12.1
		Total no. foetuses with cardiovascular malformations	CC: 0	0	1	0
Hojo (1995)	**Kimmel et al (2013)**		**0**	**10**	**100**	**300**
		Embryofetal deaths (Mean no./litter)	CC: 0.7	1.1	1.0	0.6
		Postimplantation loss (%)	CC: 7.1	13.8	8.7	6.5
		Foetuses with cardiovascular malformations (%)	CC: 0	0	1.0	0
		Foetuses with skeletal malformations (%)	CC: 0.7	3.1	4.0	5.4
		Foetuses with skeletal variations (%)	CC: 28.6	24.6	40.7*	27.7

Statistical significance vs concurrent control group: *p < 0.05 **p<0.01
CC = Concurrent control group mean HC = Historical control group range NR = None reported
^Glyphosate administered in the diet; otherwise, gavage dosing ^^Glyphosate trimesium

Brooker et al (1991b) [Reviewing Agency: BVL] The BVL assessed this study for the JMPR (2004b) and EU (1998) reviews of glyphosate. The evaluation for the EU was less detailed, but both assessments established the same NOELs / NOAELs and reached the same conclusions as to the biological significance of foetal mortality and heart malformations, based on HC data. In female rabbits orally gavaged from GD 7 – 19 at 0, 50, 150 or 450 mg/kg bw/d, there were dose-related increases in the incidence of soft / liquid faeces and inappetence and decreases in food consumption and bodyweight gain. The NOAEL for maternotoxicity was set at 50 mg/kg bw/d. Late embryonic deaths were increased significantly at 450 mg/kg, but not at the mid and low doses. At and above 50 mg/kg bw/d, total embryonic deaths and post-implantation losses were significantly higher than in the concurrent controls. Although no explicit rationale was given the BVL did not attribute embryo mortality at 50 and 150 mg/kg to treatment, possibly because the incidence of total (early + late) embryonic death was not dose-related and lay within the HC range from 21 studies performed over 1989 – 1990. The proportion of malformed foetuses was slightly increased at 150 and 450 mg/kg, due to increased incidences of interventricular septal defect and other cardiac abnormalities. However, the BVL did not consider the cardiac abnormalities to be treatment-related, as their incidences lay within the HC range in 13 studies performed in 1989. The NOAEL for developmental toxicity was set at 150 mg/kg bw/d, based on the increased incidences of late embryonic death and postimplantation loss at 450 mg/kg bw/d.

Comment: Postimplantation losses at 50 and 450 mg/kg exceeded the HC range by 2.0 and 3.5%, respectively.

Bhide and Patil (1989) [Reviewing Agency: BVL] When rabbits were gavaged with glyphosate at 0, 125, 250 or 500 mg/kg bw/d between GD 6 and 18, abortion occurred in 2/15 does from the high dose group, which also displayed depression in food consumption and bodyweight gain. A maternal NOEL of 250 mg/kg bw/d was set. Fetotoxicity, skeletal variations and visceral malformations were noted at 500 mg/kg, seen as decreased foetal viability, increased foetal non-viability and increased incidences of unilateral 14th rib, ventricular septal defect, absent kidney and absent postcaval lung lobe. A NOEL of 250 mg/kg bw/d was established for developmental toxicity. No reference was made to historical control data.

Comment: EOS's disagreement with the BVL evaluation focuses on increases in the incidences of ventricular septal defect, absent postcaval lobe and absent kidney at 125 and 250 mg/kg, even though the increases are small compared with those seen at 500 mg/kg. EOS also contends that the increase in rudimentary 14th rib at 250 mg/kg was treatment-related.

Moxon (1996b) [Reviewing Agency: BVL] This particular assessment was performed only for the JMPR review. In female rabbits orally gavaged from GD 8 – 20 at 0, 100, 175 or 300 mg/kg bw/d, the NOAEL for maternotoxicity was 100 mg/kg bw/d based on clinical signs (diarrhoea and reduced faecal output) and reduced food consumption and bodyweight gain at and above 175 mg/kg bw/d. At 300 mg/kg, mean foetal bodyweight was depressed by *ca* 8%, there were significant increases in the incidence of partially or un-ossified vertebrae and sternebrae (see Table), and slight increases in *manus* and *pes* scores[12]. The proportion of foetuses with minor skeletal defects was statistically significantly increased at the low and high doses but not at 175 mg/kg bw/d, which the BVL assigned as the NOAEL for developmental toxicity based [probably] on reduced foetal bodyweight at 300 mg/kg.

Suresh (1993a) [Reviewing Agency: BVL] Rabbits were gavaged with glyphosate at 0, 20, 100 or 500 mg/kg bw/d over GD 6 – 18. The 500 mg/kg dose caused inappetence, clinical signs, a possible depression in bodyweight gain and the death of 8/16 does. A further 4/16 does died at 100 mg/kg without displaying signs, but the BVL attributed their mortality to treatment and set the maternal NOEL at 20 mg/kg bw/d. Abortion did not occur at any dose but one doe displayed complete resorption at 500 mg/kg. At caesarean section on GD 28 there were 20 / 133, 13 / 78, 12 / 77 and 6 / 28 pregnant does / foetuses in the respective groups.

There was no treatment-related effect on external or skeletal malformations. A slight, dose-related upwards trend in the incidence of extra 13th rib was evident in the treated groups, attaining statistical significance ($p \leq 0.05$) at 500 mg/kg only. There were also eight foetuses with major visceral malformations at 500 mg/kg (significant, but p value unstated), compared with four in the control group and six at 20 and 100 mg/kg. Of these foetuses, four, four and five at 20, 100 and 500 mg/kg had dilated heart, compared with none in the control group. The percentage incidence was significant vs control ($p \leq 0.05$) at all doses; see Table. In contrast to the study author, who interpreted the lowest dose (20 mg/kg bw/d) as an effect level, the BVL reviewer assigned a NOEL of 100 mg/kg bw/d based on the increased incidence of 13th rib and heart dilation at 500 mg/kg.

[12] Pathology scores relating to the skeletal development of the hands and feet.

The BVL's rationale for the choice of NOEL was as follows:

1. The absolute number of foetuses with dilated heart was small.
2. The number of affected litters (3/13, 2/12 and 2/6 at 20, 100 and 500 mg/kg) was also low.
3. The numbers of affected foetuses or litters did not differ markedly between the treated groups.
4. The study author provided no information about the severity of heart dilation, and the consequences of such a finding in a foetus were "equivocal".
5. There was no evidence of other and much more common visceral anomalies.
6. Therefore, it was "rather unlikely" that the isolated finding of heart dilation was indeed related to treatment, but nevertheless
7. Based on the [foetal incidence data], a treatment-related effect could not be completely excluded, at least at 500 mg/kg.

Comment:

- The BVL did not identify the other major visceral malformations found in four, two, two and three foetuses at 0, 20, 100 and 500 mg/kg.
- No reference was made to HC data; hence, it is unclear whether the control group was unrepresentative of the background rates of cardiac abnormalities at the study laboratory.
- Heart dilation was classified both as a *malformation* and a major visceral *anomaly* (final paragraph of p 109 and Table B.5.6.2.2.1-1, Annex B-5). Combined with the lack of information as to the severity of the finding, this creates ambiguity as to the functional significance to the developing foetus.

Tasker et al (1980b) [Reviewing Agencies: BVL, US EPA and Australian DoHA] According to the BVL evaluation for the EU, rabbits gavaged with glyphosate at 0, 75, 175 or 350 mg/kg bw/d over GD 6 – 27 displayed clinical signs and potentially treatment-related maternal mortality at and above 175 mg/kg. The NOEL for maternotoxicity was therefore set at 75 mg/kg bw/d. There were no effects on foetal survival, growth or development, and so the foetal NOEL was set at 350 mg/kg bw/d.

The US EPA (1993) assessment differed in setting a NOAEL of 175 mg/kg bw/d for maternotoxicity. However, the EPA agreed that there was no developmental toxicity at any dose tested.

The DoHA (1985) set a NOEL for maternotoxicity at 175 mg/kg bw/d, based on diarrhoea, soft stools, nasal discharge and the death of 10/16 rabbits at 350 mg/kg. In common with the BVL and EPA, no treatment-related effects were considered to have occurred on foetal survival, growth, sex ratio or development. This assessment has been corroborated independently by Williams et al (2012).

Anon (1981) [Reviewing Agency: BVL] In this study, which the EU classified as "supplementary" due to serious reporting deficiencies, glyphosate was administered in the diet to rabbits over GD 6 – 19 at calculated actual doses of 0, 10.5, 50.7 and 255 mg/kg bw/d. There was no evidence of maternal toxicity, but foetal losses were markedly enhanced at the mid and high doses (incidences were 0.9, 0.8, 6.1 and 7.0% in the respective groups). Foetal bodyweight was not affected and no malformations were noted. The BVL assigned a NOEL of 10.5 mg/kg bw/d for fetotoxicity.

Comment: The evaluator remarked that it was unclear why "...an increase in intrauterine mortality would be elicited in a feeding study at doses far below those at which foetal effects were observed in the gavage studies. Thus, it is very doubtful whether this finding was actually related to glyphosate administration. Against the background of the data obtained in more valid, GLP-like studies, it can be concluded that the NOEL for developmental toxicity in rabbits is much higher." Presumably, the BVL reasoned that foetal exposure to glyphosate after maternal dietary dosing at 50.7 and 255 mg/kg would have been lower than attained at doses up to 350 mg/kg in the gavage studies.

Stauffer Chemical Company (1983b) [Reviewing Agency: Australian DoHA] When pregnant rabbits were gavaged with glyphosate trimesium at 0, 10, 40 or 100 mg/kg bw/d from GD 7 to 19, maternal mortality and abortion occurred at 100 mg/kg bw/d and clinical signs were observed at 40 mg/kg and above. Significant decreases in maternal bodyweight gain and food consumption were noted throughout the dosing period at 100 mg/kg, while there was depression in bodyweight during the first seven days of dosing at 40 mg/kg. The maternal NOEL was 10 mg/kg bw/d. There were no effects on foetal survival, bodyweight gain or development at any dose.

Kimmel et al (2013) [Reviewer: Scitox Assessment Services] These authors assessed seven proprietary developmental studies with glyphosate in rabbits. Five studies (Moxon, 1995b; Brooker et al 1991b, Tasker et al, 1980b; Suresh, 1993a; Bhide and Patil, 1989) had been reviewed previously by the BVL, US EPA and / or Australian DoHA (see above).

- Kimmel et al corroborated the BVL assessment of Brooker et al (1991b), describing cardiovascular malformations including intraventricular septal defect, retroesophageal right subclavian artery, dilated or narrowed aorta or pulmonary artery, and disproportionally sized ventricles, seen either alone or in combination.
- In the study of Moxon (1996b), Kimmel et al noted three foetuses (one each in the control, 100 and 300 mg/kg groups) had "heart defects involving effects on septation", together with statistically significant increases in the incidences of minor skeletal malformations at 100 and 300 mg/kg and skeletal variations at 300 mg/kg only. The NOAELs for maternal and developmental toxicity were set at 100 and 175 mg/kg bw/d, respectively, the same doses assigned by the BVL.
- Kimmel et al confirmed that there were no cardiovascular malformations or treatment-related skeletal malformations in Tasker et al (1980b), and in common with the BVL assigned a NOAEL of 75 mg/kg bw/d for maternal toxicity. Kimmel et al set a developmental NOAEL of $\geq$175 mg/kg bw/d because they considered that too few foetuses were available for adequate morphological assessment of the 300 mg/kg group.
- With respect to Suresh (1993a), Kimmel et al corroborated the BVL's reporting of maternal mortality and clinical signs but set a maternotoxicity NOAEL of 100 mg/kg bw/d. They also confirmed the BVL's stated incidences of cardiac dilation among foetuses, while adding that Suresh reported (but did not define) "seal-shaped" heart in one control foetus and one 100 mg/kg foetus, the latter also displaying cardiomegaly. Kimmel et al also clarified that two visceral malformations (single cases of liver haematoma and

absent gall bladder) seen at 500 mg/kg were unrelated to the cardiovascular system. Given that only 28 foetuses were available for examination at 500 mg/kg, Kimmel et al established the developmental NOAEL at 100 mg/kg bw/d. They commented that the observation of dilated hearts (which was unique to this study) may have been due to overly stringent inspection compared to criteria used by other laboratories.

- Kimmel et al also reviewed the study by Bhide and Patil (1989), but concluded its data were unsuitable for setting NOELs because of reporting deficiencies and inappropriate experimental methods. Nevertheless, their assessment of embryofetal mortality and malformations was consistent with the BVL's.

Two other studies in rabbits (Hojo, 1995; Coles and Doleman, 1996) have not been included in any available agency review. Hojo administered glyphosate by oral gavage at 0, 10, 100 or 300 mg/kg bw/d over GD 7 – 19 and observed hypoplasia of the pulmonary artery and ventricular septal defect in one foetus at 100 mg/kg, but no other cardiac abnormalities. No skeletal variations or malformations were ascribed to treatment. Based on clinical signs (soft / liquid faeces) at 300 mg/kg, a NOAEL of 100 mg/kg bw/d was assigned for maternal toxicity. The developmental NOAEL was $\geq$300 mg/kg bw/d.

Coles and Doleman gave oral gavage doses of 0, 50, 200 or 400 mg glyphosate/kg bw/d to pregnant rabbits from GD 7 to 19. Based on clinical signs (soft, liquid, mucoid faeces) and decreased bodyweight gain, a NOAEL for maternal toxicity was set at 200 mg/kg bw/d. Embryofetal deaths and post-implantation losses were increased at 200 and 400 mg/kg, but statistical significance was attained at 200 mg/kg only. At 400 mg/kg, the increase was due to one doe with nine late foetal deaths, which Kimmel et al considered to be of questionable biological significance. At 200 mg/kg, a heart and great vessel defect occurred in an acephalic (headless) foetus. However, there were no other cardiovascular malformations and no treatment-related skeletal malformations or variations. A NOAEL of $\geq$400 mg/kg bw/d was assigned for developmental toxicity.

After Kimmel et al aggregated the data for each dose level (excluding those from Bhide and Patil, 1989), the incidences of septum-related defects were 1/770 in controls and 6/1939 among glyphosate-exposed foetuses (i.e. 0.13 and 3.1%). Four of the six cases in treated groups occurred at the maternally toxic dose of 450 mg/kg. Septal defects were *not* observed among 747 foetuses whose mothers received 175, 200, 300, 350 or 400 mg/kg bw/d.

Cardiomegaly was seen in one foetus at 100 mg/kg (i.e. in 1/374 foetuses or 0.27% incidence), while one case of dilated ventricles occurred at 0, 100 and 500 mg/kg (i.e. 1/770, 1/374 and 1/28 fetuses in the respective groups, = 0.13, 0.27 and 3.6% incidences). Dilated heart was reported in 4/78 (5.1%), 4/374 (1.1%) and 5/28 (17.9%) foetuses at 20, 100 and 500 mg/kg. None of the 954 foetuses whose mothers received glyphosate at 150 – 450 mg/kg bw/d displayed cardiac or ventricular enlargement or dilation. The aggregated data suggest that even if they are not a reporting artefact, the cases at 20 and 100 mg/kg bw/d were not treatment-related.

Kimmel et al concluded that "there was no increase in cardiovascular malformations at doses that were not overtly toxic to the pregnant rabbits (i.e. generally at doses over 150 mg/kg [bw]/d").

Comment: Inclusion of data from Bhide and Patil (1989) in the aggregated dataset would make negligible difference to Kimmel et al's analysis of the incidences of cardiac / ventricular enlargement or dilation, since these findings were reported only by Suresh (1993a). It would add single cases of septal defects at 125 and 250 mg/kg and a further two cases at 500 mg/kg bw/d, making a total of ten affected foetuses from treated mothers[13] (one each at 100, 125, 150 and 250 mg/kg, with four at 450 and two at 500 mg/kg). In the APVMA's opinion, this pattern is most consistent with septal defects having a relationship to treatment at 450 and 500 mg/kg, but not at $\leq$250 mg/kg bw/d.

[13] Kimmel et al do not report the numbers of foetuses Bhide and Patil (1989) examined at each dose, so the incidences of septal defects in all seven rabbit studies combined are unknown.

APPENDIX 3: ASSESSMENTS OF REPRODUCTIVE TOXICITY STUDIES

A3.1 Rats

The German BVL has evaluated eight reproduction studies on glyphosate in rats, of which six were included only in the EU (1998) review, one appeared in the JMPR (2004b) review, and the remaining study was assessed in both reviews. The toxicological end-points examined included oestrus cycling, mating performance, pregnancy rate, gestation length, numbers, sexes, growth, post-natal developmental landmarks and onset of puberty in pups, bodyweights, histology of the reproductive organs and analysis of sperm and oocytes.

Moxon (2000) [Reviewing Agency: BVL] The study was performed over two generations at dietary glyphosate concentrations of 1000, 3000 and 10 000 ppm. In the JMPR review, the BVL found no effects on sexual development or fertility at up to the highest dietary concentration of 10 000 ppm (985 mg/kg bw/d). A NOAEL for parent and offspring toxicity was set at 3000 ppm (293 mg/kg bw/d) based on a reduction in bodyweight of F1A pups and a subsequent reduction in bodyweight of F1 parent males at 10 000 ppm.

Brooker et al (1992) [Reviewing Agency: BVL] This was a two-generation study performed at dietary glyphosate concentrations of 1000, 3000 and 10 000 ppm in the diet. For the EU review, the BVL based a NOEL for parental toxicity of 1000 ppm (79 and 87 mg/kg bw/d in males and females) on histological abnormalities in the parotid and submaxillary salivary glands at glyphosate dietary levels of 3000 and 10 000 ppm. A NOEL of 10 000 ppm (*ca* 797 and 881 mg/kg bw/d in males and females) was set for effects on reproduction and pups.

In the JMPR review, the BVL concluded that there had been no effects on sexual development or fertility at up to the highest dietary concentration of 10 000 ppm. A NOAEL of 3000 ppm (197 mg/kg bw/d) for parent and offspring toxicity was assigned based on increased food and water consumption in F1 females, depressed bodyweight in F1 males, and an increased incidence of cellular alteration of the salivary glands in F0 and F1 adults at 10 000 ppm[14].

Brooker et al (1991c) [Reviewing Agency: BVL] Prior to the main study (above), a one generation range finding experiment was performed on small numbers of rats at dietary glyphosate concentrations of 0, 3000, 10 000 and 30 000 ppm. The parental generation received treatment from GD 3 to PND 21, after which their offspring were treated until termination a six weeks of age. Fecundity and pup survival were unaffected, but [unquantified] reductions in pup bodyweight occurred at all doses. Hence, a NOEL was not established. The BVL discounted this finding because none of the fully comprehensive reproduction studies reviewed for the EU had found treatment-related effects on pups at up to and including 10 000 ppm.

[14] The discrepancy between the BVL's conclusions for the EU and JMPR reviews occurred because the JMPR assigns No Observed *Adverse* Effect Levels to toxicology studies, as opposed to No Observed Effect Levels (as assigned by the EU and Australia). By JMPR criteria, the histological abnormalities in the salivary glands at 3000 ppm were not classified as an adverse effect.

Reyna (1990) [Reviewing Agencies: BVL and US EPA] A two-generation study was performed at dietary levels of 0, 2000, 10 000 and 30 000 ppm. In-life and *post mortem* examinations conformed with OECD TG 416 and included histological examination of reproductive organs from all control and high dose F0 and F1 adults and one F2B weanling/sex/litter. A NOEL of 10 000 ppm (722 and 757 mg/kg bw/d for males and females, respectively) was assigned for parental and offspring toxicity. This was based on reduced bodyweight gain and soft faeces in adults receiving 30 000 ppm, and reductions in litter size and pup bodyweight gain during lactation at this same dietary level.

The US EPA (1993) evaluation of Reyna (1990) differed slightly from the EU / BVL assessment insofar as there was no mention of decreased litter size, but was otherwise closely similar. The EPA assigned a systemic NOEL of 10 000 ppm (500 mg/kg bw/d), a reproductive NOEL of 30 000 ppm (1500 mg/kg bw/d) and a developmental NOEL of 10 000 ppm (500 mg/kg bw/d). The doses appear to have been estimated, rather than having been calculated from parental food intake.

Suresh (1993b) [Reviewing Agency: BVL], In this two-generation study compliant with OECD TG 416, there were no treatment-related effects on the parents or offspring at the highest administered dietary level of 10 000 ppm, equivalent to *ca* 700 – 800 mg/kg bw/d. The BVL therefore set a NOEL of 10 000 ppm.

Antal (1985) [Reviewing Agency: BVL] Similarly, the BVL assessed this three-generation study as having demonstrated no effects of treatment at the highest dietary concentration of 5000 ppm in the diet, or 462 and 502 mg/kg bw/d in males and females. A NOEL of 5000 ppm was therefore assigned for parental and reproductive toxicity.

Bhide (1988b and 1988c) & Schroeder and Hogan (1981) [Reviewing Agencies: BVL, US EPA and Australian DoHA] These three studies were performed at very low doses, and the BVL / EU regarded them as providing supplementary information only. No treatment-related effects occurred in the parental generations or offspring in a three-generation study at dietary feeding levels of 0, 75, 150 and 300 ppm, equivalent to *ca* 15 mg/kg bw/d at the high dose (Bhide, 1988b); during a single-generation study by oral gavage at 0, 5 and 10 mg/kg bw/d prior to mating, through pregnancy and up to PND 21 (Bhide, 1988c); or in a three-generation dietary study at 0, 3, 10 and 30 mg/kg bw/d (Schroeder and Hogan, 1981).

The DoHA (1985) and the US EPA (1993) also assessed Schroeder and Hogan (1981) as having demonstrated no treatment-related effects on the parental or filial generations, and set a NOEL of 30 mg/kg bw/d. This NOEL forms the basis for the current Australian ADI for glyphosate, of 0.30 mg/kg bw/d. A DoHA (1992) evaluation reached the same conclusions as the BVL with regard to the studies by Bhide (1988b and 1988c).

Stauffer Chemical Company (1983a) [Reviewing Agency: Australian DoHA] In a two-generation study with glyphosate trimesium in rats at dietary concentrations of 0, 150, 800 and 2000 ppm (equivalent to *ca* 7.5, 40 and 100 mg/kg bw/d), the only adverse effect on reproductive indices was a reduction in litter size at 2000 ppm. A NOEL of 150 ppm was assigned for the parental animals and offspring based on reduced bodyweight gain, food consumption and plasma protein and albumin levels in

adults and depressed pup bodyweight and relative spleen weight at and above 800 ppm (DoHA, 1991).

APPENDIX 4: STUDY ASSESSMENTS PERFORMED BY MARK JENNER, SCITOX ASSESSMENT SERVICES

A4.1 Effects of a glyphosate-based herbicide formulation on gene expression in vitro

Hokanson et al (2007): In a study of the effects of glyphosate on the expression of oestrogen-regulated genes, MCF-7 human breast adenocarcinoma (oestrogen sensitive) cells were exposed to an unidentified home garden herbicide containing 15% glyphosate (no additional details provided) with or without 3.0×10^{-10} M 17β-estradiol (oestrogen). Cells were incubated for 18 hours with the herbicide at final glyphosate concentrations of 0.23, 0.023, 0.0023, or 0.00023%. Following purification of cellular RNA and generation of cyanine 3- and 5-labelled anti-sense RNA, the activity of 1550 genes was then measured by DNA microarray analysis using RZPD chips.

According to the study authors, 680 of the 1550 investigated genes were dysregulated by exposure to the herbicide. However, they did not state by how much the affected genes' activity differed from control levels, or at what glyphosate concentrations. The study authors listed a sub-set of 29 genes whose activities were up- or down-regulated by greater than 2-fold, of which seven were tested further by quantitative real-time PCR to corroborate the results of DNA microarray analysis.

Only three of the 1550 genes fulfilled the criteria for significant dysregulation, when appraised by both methods. In the presence of glyphosate at 0.00023%, DNA microarray analysis indicated that HIF1 was up-regulated by 2.2-fold, while CXCL12 and EGR1 were down-regulated to 0.46 and 0.49 of control activity. qrtPCR expression analysis showed that HIF1 was up-regulated by over two-fold whereas CXCL12 and EGR were down-regulated by over 50%. For each gene, cell treatment with oestrogen alone yielded expression levels that were intermediate between those observed in control cells and cells exposed to oestrogen and herbicide combined.

According to the study authors, the HIF1 gene primes cells for the initiation of apoptosis under hypoxic conditions, and therefore plays a key role in cell death resulting from cerebral and myocardial ischemia. They raise the possibility that elevated levels of HIF1 [protein] may initiate apoptosis in the absence of hypoxia, promoting a variety of hypoxia-initiated patho-physiological states including ischemia of the myocardium, brain and retina; pulmonary hypertension, pre-eclampsia and intrauterine [foetal] growth retardation.

The CXCL12 gene product (also known as stromal cell-derived factor 1 and pre-beta cell growth-stimulating factor) is a lymphocyte chemoattractant, may be involved in lymphocyte activation, and is reportedly critical for the mobilisation of cells of the haematopoietic tissues into peripheral blood. Hokanson et al suggest that altered [decreased] levels of CXCL12 may contribute to disruption of immune surveillance and basal extravasation of mono- and lymphocytes.

Among the biological effects attributed to EGR1 are regulating the expression of transforming growth factor beta-1, involvement in the suppression of [cellular] growth and transformation, and the regulation of apoptosis, endothelial cell growth, neovasculatisation, tumour initiated angiogenesis and tumour growth. The study authors consider that [decreased] levels of EGR1 may potentially affect the rate of

initiation of apoptosis and alter the level of vascularisation associated with tumour formation.

Comment

This paper is of limited value: it does not identify which components of the glyphosate-based herbicide formulation are responsible for altering gene expression, does not identify any mode of action of those components, does not provide evidence that the observed changes in gene expression are anything other than homeostatic regulation, and does not establish that the effects observed in MCF-7 cancer cells in vitro would be representative of those that would occur in non-cancerous mammalian cells (especially within tissues or at the whole animal level). Other than retardation of foetal growth, the postulated effects of HIF1, CXCL12 and EGR dysregulation have not been reported in toxicology studies in laboratory animals, and there appears to be no justification for extrapolating from the study's findings to predicting adverse effects on human health.

Mink et al (2011) have reviewed epidemiological studies relevant to some of the non-cancer end-points that Hokanson et al speculate may be affected. In the study populations, there was no statistically and/or biologically association between exposure to glyphosate and retinal degeneration (Kirrane et al, 2005), myocardial infarction (Dayton et al, 2010 and Mills et al, 2009) or depressed birthweight (Sathyanarayana et al, 2010). Furthermore, epidemiological evidence of associations between glyphosate exposure and cancer is weak and conflicting (DoHA, 2005). A recent review (Mink et al, 2012) of epidemiological studies relevant to cancer end-points considered seven cohort studies and fourteen case-control studies looking at possible associations between glyphosate and one or more cancer outcomes; there was no consistent pattern of positive associations to indicate any causal relationship between total cancer (in adults or children) or any site-specific cancer and exposure to glyphosate.

A4.2 Cytotoxicity of glyphosate, AMPA and glyphosate-based herbicides in vitro

Benachour et al (2007): Human embryonic kidney (HEK) 293 and human choriocarcinoma-derived placental JEG3 cells were exposed for 1 – 72 hours *in vitro* to Roundup Bioforce (360 g/L glyphosate acid present as 480 g/L glyphosate isopropylamine salt, no other constituents identified; Monsanto, Anvers, Belgium) at up to 2% in the incubation medium, or glyphosate at equivalent concentrations (up to 42 mM). Cell viability was measured by the MTT assay, based on the cleavage of MTT by the mitochondrial enzyme succinate dehydrogenase (SDH). When the effects of the test formulation and glyphosate were compared, glyphosate solutions were adjusted to *ca* pH 5.8, the pH of a 2% Roundup solution.

Roundup Bioforce showed greater concentration- and time-dependent cytotoxicity against both cell lines than glyphosate at equivalent concentrations, suggesting that adjuvants in the formulation were contributing to cellular injury. JEG3 cells were more resistant to Roundup Bioforce than HEK293 cells, but both types were of similar susceptibility to glyphosate.

Table 4.1: EC50s* (% in serum-containing medium) of Roundup Bioforce and equivalent concentrations of glyphosate for viability of HEK293 and JEG3 cells.

Test compound	1 h	24 h	48 h	72 h
	HEK293 cell line			
Roundup Bioforce	1.4	0.8	0.7	0.05
Glyphosate	>>2.0	1.7	1.7	1.5
	JEG3 cell line			
Roundup Bioforce	>>2.0	1.3	0.4	0.2
Glyphosate	>>2.0	1.8	1.5	1.5

*EC50 (not the LD50 as claimed by the study authors[15]) = the concentration required to cause a 50% decrease in mitochondrial SDH activity. As the data were provided in graph form, all values are approximate.

Effects of Roundup Bioforce and glyphosate on the activity of aromatase (CYP19; an enzyme catalysing the conversion of androgens to oestrogens) were measured in HEK293 cells transfected with human aromatase cDNA, human placental cell microsomes and equine testicular microsomes. The HEK293 cells were exposed to the test compounds for 24 hours at up to 0.2% Roundup or 1% glyphosate, while microsomes had a 15-minute exposure period at up to 10% Roundup or 2% glyphosate. The assay quantified the release of tritiated water from [1β-^{3}H]-androstenedione.

Both the formulation and active constituent weakly inhibited aromatase activity *in vitro*. Under pH-adjusted conditions at 37 ^{o}C, glyphosate had IC50s of *ca* 1.0% and 0.8% against aromatase in placental microsomes and HEK293 cells, respectively. Over its tested concentration range (0.01 – 0.2%), Roundup Bioforce inhibited aromatase by *ca* 20% in HEK293 cells. Roundup Bioforce had an IC50 of *ca* 4% against aromatase activity in human placental and equine testis microsomes, at 25 ^{o}C and physiological pH.

Comment

The concentrations of Roundup and glyphosate required for cytotoxicity and aromatase inhibition were similar to those present in herbicidal spray mixtures (1 – 2% formulation or 21 – 42 mM glyphosate), orders of magnitude higher than would be attained within cells or tissues in vivo under physiological conditions. Over the more biologically relevant concentration range 0.001 – 100 µM, glyphosate has no effect on steroid hormone production in the H295R steroidogenesis assay, developed by the OECD as an in vitro screening assay for endocrine disrupting chemicals (Hecker et al, 2010). Given that surfactants inhibit aromatase activity by disrupting mitochondrial membranes (Levine et al, 2007), the reported effects of Roundup Bioforce in HEK293 cells and microsomes are likely to be experimental artefacts. Another confounding factor would have been the pH of the incubation medium, which was below the physiological range during the cell viability assays.

Benachour and Seralini (2009) evaluated the *in vitro* cytotoxicity of glyphosate (Sigma-Aldrich), the glyphosate metabolite AMPA (Sigma-Aldrich), four glyphosate-based herbicide products (see table below), and the surfactant polyethoxylated tallow amine (POEA; a component of some glyphosate formulations) to human umbilical

[15] Cellular viability was not quantified, so it could not be confirmed that the "LD50" actually corresponded to the death of half the population of exposed cells.

cord vein endothelial cells (HUVEC)[16] and the human choriocarcinoma-derived placental (JEG3) and human embryonic kidney (HEK293) cell lines.

Table 4.2: Glyphosate-based herbicides studied in Benachour & Seralini (2009) All products were manufactured by Monsanto, Anvers, Belgium

Product Name (Abbreviation used in evaluation)	Glyphosate concentration (g/L)
Roundup Express (R7.2)	7.2
Roundup Bioforce* (R360) Roundup Extra 360*	360
Roundup Grands Travaux (R400)	400
Roundup Grands Travaux Plus (R450)	450

*The study authors treated both products as being the same formulation.
No further information on product composition was provided.

Cells were exposed for 24 hours in serum-free medium to each individual test compound at 14 concentrations ranging from 10 ppm to 20 000 ppm (0.001% to 2%). Cells were also exposed to POEA at 1 and 5 ppm, and AMPA at 4, 6, 8 and 10%. Using sub-toxic concentrations of glyphosate, AMPA and POEA, evidence of additive or synergistic toxicity was sought in HEK293 and JEG3 cells exposed to combinations of POEA 1 ppm + glyphosate or AMPA 5000 ppm, and glyphosate 4000 ppm + AMPA 1000 ppm. HUVEC cells were exposed to POEA 1 ppm + glyphosate or AMPA 500 ppm, and glyphosate 400 ppm + AMPA 100 ppm.

After incubation, cytotoxicity was assessed by the following criteria: *Adenylate kinase (AK) activity* in the incubation medium, as a biomarker of cytoplasmic membrane rupture (assumed to result from cellular necrosis, either primary or secondary after apoptosis); *Intracellular succinate dehydrogenase (SDH) activity*, assayed by the MTT test as a measure of mitochondrial respiration rate; and *Intracellular caspase 3/7 activity*, as indicators of apoptosis. Results from the cytotoxicity assays were presented in graphical form alone, and therefore only approximate quantitative values are available.

Results

Cytotoxicity, assessed by impact on mitochondrial respiration rate: In all three cell types, the concentration of glyphosate causing a 50% decrease in SDH activity (ie, the EC50, and not the LD50 as claimed by the study authors[17]) was *ca* 10 000 ppm. The metabolite AMPA was markedly less toxic, having EC50s of *ca* 40 000, 100 000 and >100 000 ppm in HEK293, JEG3 and HUVEC cells, respectively. By contrast, POEA was highly cytotoxic, demonstrating a lowest EC50 of *ca* 3 ppm (see following table). All Roundup formulations were more toxic than the active constituent. Moreover, their EC50s were not linearly proportional to the concentration of glyphosate in the products or incubation medium. This is consistent with other formulation components being cytotoxic and/or potentiating the toxicity of the active constituent.

[16] HUVEC cells were chosen because *in vivo*, they form a permeable barrier between the blood and the underlying tissues and would be exposed directly to circulating chemicals, for which they may be a target.
[17] Cellular viability was not quantified, so it could not be confirmed that the "LD50" actually corresponded to the death of half the population of exposed cells.

Table 4.3: Concentrations of glyphosate and other test compounds causing a 50% decrease in intracellular succinate dehydrogenase activity in HUVEC, JEG3 and HEK293 cells

Test compound	Approx EC50 (ppm)	Glyphosate concentration (ppm) in medium at the EC50
Glyphosate	10 000	10 000
AMPA	≥40 000	-
POEA	3 – 30	-
R 7.2	6000 – 9000	42 – 63
R360	2000 – 3000	720 – 1080
R400	30	12
R450	100	45

Cell membrane integrity: AMPA, POEA and the Roundup formulations caused increases in extracellular AK activity, consistent with leakage or rupture of cell membranes. By contrast, cells exposed to glyphosate alone released little or no AK, even in the presence of marked depression in mitochondrial respiration. The study authors interpreted this as evidence that glyphosate does not mediate cell death by necrosis, in contrast to AMPA, POEA and Roundup formulations.

Interactions between glyphosate, AMPA and POEA, assessed by effects on cell membrane integrity: Combinations of glyphosate + POEA, glyphosate + AMPA and AMPA + POEA (see above) were clearly more cytotoxic to HUVEC and HEK293 cells than the individual chemicals, causing about 2-fold and 4 to 8-fold more extensive release of AK from the two respective cell types. However, for reasons unknown, additive or synergistic toxicity was not observed in JEG3 cells.

Apoptosis: At incubation concentrations of 50 ppm and above, glyphosate and R360 induced transient but marked increases in intracellular caspase 3/7 activity within HUVEC cells. The effect was first observed after 6 hours of exposure. After 12 hours, caspase activity peaked at 20 – 30 times control levels. Reversibility was well advanced by 18 hours and complete at 24 hours. Similar but much weaker responses occurred in HEK293 and JEG3 cells, within which caspase 3/7 activity increased by no more than 2 or 3-fold. These cell lines were markedly less sensitive than HUVEC cells, requiring glyphosate and R360 concentrations of at least *ca* 10 000 and 1000 ppm, respectively, for induction of caspase activity. Cell death, loss of adhesion, shrinkage and fragmentation were confirmed microscopically in all cell types after 24 hours exposure to 50 ppm R400. DAPI staining revealed DNA condensation in HUVEC, HAK293 and JEG3 cells exposed to glyphosate or R360 at 5000 ppm.

No findings were presented on the influence of AMPA and POEA on caspase activity or cell morphology.

Comment

The French Agency for Food Safety (AFSSA, 2009) has reviewed Benachour and Seralini (2009), commenting that:

- During exposure to the test compounds, cells were incubated for 24 hours in medium without serum, which could lead to disturbance of their physiological state.

- The glyphosate tested in the study was glyphosate acid, whereas glyphosate isopropylamine salt was present in the commercial formulations tested. No precise information regarding pH was given, except at the highest concentrations [where the pH was adjusted to 5.8].
- Cytotoxicity and induction of apoptosis may have been due to pH and / or variations in osmotic pressure at the highest concentrations tested.
- Surfactant effects and increased osmolality are known to increase membrane permeability, causing cytotoxicity and induction of apoptosis.
- The test cells were exposed at extremely high concentrations of the test compounds under physiologically abnormal conditions.

A4.3 Cytotoxicity, anti-estrogenic and anti–androgenic activity, and genotoxicity of glyphosate and glyphosate-based herbicides in vitro

Gasnier et al (2009) assessed the activity of glyphosate and four glyphosate-based herbicides (R7.2, R360, R400 and R450; see above evaluation of Benachour and Seralini (2009)) in the HepG2 human hepatoma or MDA-MB453-kb2 cell lines. The following end-points were investigated:

Cytotoxicity: *Intracellular SDH activity*, *extracellular AK activity* and *intracellular caspase 3/7 activity* were measured in HepG2 cells as described by Benachour and Seralini (2009). Cell viability was also assessed by the *Alamar Blue assay* and the *neutral red assay*, following 24 hours of exposure to the test compounds over the range 10 – 20 000 ppm.

Anti-oestrogenic activity:
(a) *The activity of aromatase,* the enzyme responsible for converting androgens to oestrogens, was measured in HepG2 cells after 24 hours of exposure to "non-toxic" concentrations of glyphosate or R7.2, 360, 400 and 450. The assay was based on the release of tritiated water from [1β-^{3}H]-androstenedione. *Aromatase mRNA levels* were also assayed, by semi-quantitative reverse transcriptase-PCR.

(b) *Activity at human oestrogen receptors* was measured in HepG2 cells transfected with hERα and hERβ and then incubated with 17β-estradiol (at 10^{-8}M) and glyphosate or R7.2 (each at up to 3000 ppm), R360 (up to 2000 ppm), R400 (up to 10 ppm), R450 (up to 30 ppm) or the positive control ICI 182x780 (at 10^{-8}M).

Anti-androgenic activity was measured in MDA-MB453-kb2 human breast cancer cells (which possess a high level of androgen receptor) incubated for 24 hours with glyphosate (up to 1500 ppm) or R7.2, (up to 2000 ppm), R360 (up to 500 ppm), R400 (up to 2 ppm) or R450 (up to 40 ppm) plus DHT (4×10^{-10} M). The positive control was nilutamide (10^{-6} M).

Genotoxicity: Single- and double-stranded DNA breakage and alkali-labile DNA damage were investigated in HepG2 cells after 24 hours of exposure to R400 at 1, 2.5, 5, 7.5 and 10 ppm, using the single-cell gel electrophoresis (Comet) assay. Benz[a]pyrene (50 µM) was used as positive control. It is unclear whether glyphosate or other Roundup formulations were tested.

Results

Cytotoxicity: SDH and AK activity and the Alamar Blue assay yielded fairly consistent results in the experimental system employed. As shown in the following table, the absolute and relative cytotoxic potencies of glyphosate and Roundup formulations against HepG2 cells were similar to those described by Benachour and Seralini (2009) against other human cell lines *in vitro*. Again, Roundup formulations were moderately – markedly more toxic than the active constituent, and their relative potency was not proportional to the concentration of glyphosate they contained.

Table 4.4: LOECs or EC50s of glyphosate and Roundup formulations against indices of cytotoxicity in HepG2 cells.

Test compound	Alamar Blue assay		SDH inhibition		AK activity
	LOEC (ppm)	EC50* (ppm)	LOEC (ppm)	EC50* (ppm)	LOEC (ppm)
Glyphosate	10 000	27 800	10 000	18 000	>20 000
R 7.2	2000	3600	8000	8600	8000
R360	1000	2200	5000	6500	3000
R400	5	12	50	55	50
R450	50	60	80	170	60

*Reported as LC50

At 60 ppm, R450 formulation induced apoptosis in HepG2 cells, seen as a 156% increase in caspase 3/7 activity following 24 hours exposure ($p<0.05$ vs control) and a 765% increase after 48 hours ($p<0.01$). No further data on apoptotic activity were presented.

Anti-oestrogenic activity: Over the range 600 – 3000 ppm, glyphosate had no statistically significant effects on aromatase transcription and activity in HepG2 cells, and was also devoid of anti-oestrogenic activity at hERα and β.

By contrast, intracellular aromatase activity was significantly ($p<0.05$ or <0.01) inhibited in the presence of Roundup formulations. R7.2 caused *ca* 75% inhibition at 8000 ppm. R360, R450 and R400 caused no more than *ca* 50% inhibition of aromatase activity, but maximal inhibition occurred at lower concentrations ($\geq$800, 50 and $\geq$10 ppm respectively). The mode of inhibition was not elucidated but is unlikely to have depended on inhibition of DNA transcription, because aromatase mRNA levels were generally increased in Roundup-exposed cells.

All Roundup formulations dose-dependently inhibited oestrogen-dependent transcription in HepG2 cells. R7.2 and R360 were the least potent, with IC50s of *ca* 1500 – 2500 ppm, whereas R400 and R450 had *ca* 100 – 500 times greater potency (see following table). Anti-oestrogenic potency was not correlated with the concentration of glyphosate present in the formulations or cell incubation medium.

Anti-androgenic activity: Roundup formulations dose-dependently inhibited androgen-dependent transcription in MDA-MB453-kb2 cells. R7.2 and R360 were the least potent, with respective IC50s of *ca* 800 and 300 ppm, whereas R400 and R450 had *ca* 10 – 100 times greater potency (see following table). Anti-androgenic potency was independent of glyphosate concentration.

The study authors claimed that glyphosate "was clearly anti-androgenic at sub-agricultural and non-cytotoxic dilutions". This is, however, open to question: androgen receptor-mediated transcriptional activity was depressed by *ca* 30% at the lowest glyphosate concentration tested (100 ppm?), 45% at 500 ppm but only 20% at 1500 ppm (data were presented graphically, so all values are approximate). Although

the difference from control was statistically significant ($p<0.01$) at all three concentrations, the lack of dose-dependency and failure to attain 50% inhibition are remarkable, inconsistent with the behaviour of the Roundup formulations, and seem inconsistent with a receptor-mediated phenomenon. Furthermore, results obtained with the positive control were not presented.

Table 4.5: IC50s of Roundup formulations against human steroid receptors, expressed as ppm formulation (upper line) and µM glyphosate (lower line) in the cell incubation medium

Receptor	R7.2	R360	R400	R450
hERα	2030 ppm 86.5 µM	1450 ppm 3088 µM	6.0 ppm 14.2 µM	20 ppm 53.2 µM
hERβ	2460 ppm 105 µM	1600 ppm 3407 µM	3.0 ppm 7.1 µM	ND ND
hAR	770 ppm 32.8 µM	310 ppm 660 µM	0.9 ppm 2.1 µM	20 ppm 53.2 µM

hERα = human oestrogen receptor α **hERβ** = human oestrogen receptor β
hAR = human androgen receptor ND = No data

Genotoxicity: R400 caused a dose-dependent increase in DNA strand breaks[18]. Compared with the negative control (35% breakage, with 15% class 1, 10% class 2 and 10% class 3 breaks), there was *ca* 50% total breakage at 5 ppm (comprising 25% class 1, 11% class 2 and 15.5% class 3 breaks), 60% breakage at 7.5 ppm and 75% breakage at 10 ppm (*ca* 13% class 1, 27% class 2 and 36% class 3 breaks). The NOEC was 2.5 ppm. The positive control caused 95% total breakage, of which *ca* 70% consisted of class 3 breaks.

However, there results were not necessarily caused by genotoxic activity. In the Alamar Blue assay (the most sensitive index of cytotoxicity), R400 was toxic against HepG2 cells at concentrations of 5 ppm upwards, with an EC50 of 12 ppm. It is therefore possible that the increased DNA strand breakage seen at 5 – 10 ppm arose from cellular injury or death, rather than from direct damage to DNA.

Comment

The study did not demonstrate whether the observed inhibition of aromatase and steroid receptor-mediated transcription was caused by glyphosate or other components in the test products. If surfactants were present, it is highly probable that they contributed to these effects, given that surfactants interfere with *in vitro* assays for aromatase activity and steroidogenesis (US EPA, 2009; Levine et al, 2007; & DeSesso and Williams, 2012).

Clair et al (2012): The study authors measured the cytotoxicity of glyphosate and a glyphosate-based herbicide, and investigated their effects on testosterone production and oestrogen and androgen receptor mRNA levels in rat testicular cells *in vitro.* The test compounds were laboratory-grade glyphosate (Sigma-Aldrich, Saint-Quentin Fallavier, France) and Roundup Bioforce (360 g/L glyphosate acid; no other information provided). Stock solutions of glyphosate (7.6 g/L) or 2% Roundup (= 7.6 g glyphosate/L) were prepared in cell culture medium and diluted as required.

[18] Class 1 = minimum damage, Class 2 = medium and Class 3 = maximum

Leydig, Sertoli and germ cells were isolated and purified from the testes of 70-day-old Sprague-Dawley rats. Leydig cells were incubated for 1 – 48 hours with Roundup at 0.005 – 1.0% in solution or equivalent concentrations of glyphosate. The other cell types appear to have been exposed to the same range of concentrations for 24 or 48 hours. Cytotoxicity was assessed by measurement of adenylate kinase (AK) activity (an index of cytoplasmic membrane rupture) in cell supernatants using the ToxiLight bioassay. To measure the extent of apoptosis, intracellular caspase 3 / 7 activity was quantified by the Caspase-Glo assay, and nuclear DNA was visualised *in situ* by DAPI fluorescence staining.

In Leydig cells that had been exposed for 24 hours to glyphosate or Roundup at 0.0001 – 0.10%, 3β-hydroxysteroid dehydrogenase (3β-HSD) activity was measured as an index of testosterone synthesis, and the testosterone concentration in the cell culture medium was quantified by RIA. mRNA expression of aromatase, AR, HERα and HERβ was measured by real-time PCR.

Results

Cytotoxicity (cell lysis): In Leydig cells, glyphosate caused no increase in AK activity over the concentration and time range tested, suggesting a lack of necrosis associated with cytotoxicity. By contrast, cytotoxicity was evident after one hour of exposure to Roundup at ≥0.10%. The peak effect (*ca* 3-fold increase in AK activity vs unexposed controls) occurred from 3 – 24 hours at concentrations between 0.50 and 1.0% ($p<0.005$ or 0.001).

Germ cells were resistant to injury by glyphosate (no increase in AK activity seen) and comparatively insensitive towards Roundup, which caused a maximum of *ca* 20% increase in AK activity at 24 hours at 0.50% ($p<0.001$) and at 48 hours at 0.005% ($p>0.05$).

Glyphosate was cytotoxic towards Sertoli cells, eliciting *ca* 2-fold increases in AK activity at 24 hours at 0.01 and 0.05% ($p>0.05$). Roundup also injured Sertoli cells by 24 hours, but the peak effect (a 2-fold increase in AK activity) occurred at 0.10% ($p<0.05$).

Apoptosis: In the time course experiment with Leydig cells, the only evidence of caspase activation was seen after six hours exposure to Roundup at 0.05%, which elicited a *ca* 15% increase in activity ($p<0.01$). Over the 0.1% - 1.0% concentration range, by contrast, Roundup caused concentration-dependent *decreases* in caspase activity from one hour onwards, with almost complete loss of activity after 12 – 48 hours' exposure at ≥0.5% ($p<0.001$). Roundup caused a similar effect in Sertoli and germ cells after 24 hours of exposure.

In contrast to the formulation, glyphosate did activate caspase in Leydig cells. Relatively weak (10 – 20%) and inconsistent increases in activity were observed from six hours onwards at concentrations of 0.005% and above. In germ cells, 0.005 and 0.01% glyphosate increased caspase activity by *ca* 20% after 24 hours exposure ($p<0.01$), while 20 – 40% increases ($p<0.01$ to 0.001) in activity were evident at 48 hours over the concentration range 0.50 – 1.0%. Glyphosate did not, however, mediate any consistent effect on caspase activity in Sertoli cells.

Morphological evidence of apoptosis (compaction of chromatin and DNA within the nucleus) was observed in Leydig cells exposed for 24 hours to Roundup at 0.05 and 1.0%, or glyphosate at 1.0%. However, there was no comment as to whether nuclear condensation also occurred in Sertoli or germ cells.

Testosterone: Neither Roundup nor glyphosate influenced the 3β-HSD activity in Leydig cells exposed for 24 hours at 0.0001 – 0.10%. Testosterone concentration in the cell incubation medium was depressed by *ca* 1/3rd ($p<0.01$) by glyphosate and Roundup at 0.0001%, but not at or above 0.005%.

Expression of aromatase, AR, HERα and HERβ in Leydig cells: Aromatase mRNA levels increased by *ca* 7.5-fold ($p<0.005$) in response to a 24-hour exposure to glyphosate at 0.001%, but rose by only 2-fold at 0.005 and 0.01% (non-significant). A non-significant, three-fold increase in aromatase mRNA occurred following exposure to Roundup at 0.001%, but at 0.005 and 0.01% there was no effect. Aromatase activity and oestrogen levels were not measured. Neither glyphosate nor Roundup had any effect on androgen or oestrogen receptor mRNA levels under the experimental conditions.

A4.4 Developmental and reproductive effects of glyphosate-based herbicide in amphibians and birds

Paganelli et al (2010) performed studies on neural crest development in three experimental systems:

(i) *Xenopus laevis* embryos, which were exposed from the 2-cell stage onwards to Roundup Classic (a Monsanto product containing 48% w/v of an unspecified glyphosate salt; no other constituents were identified) at 3000-, 4000- and 5000-fold dilutions in their incubation medium. The final concentrations of glyphosate were 717, 536 and 430 µM at the respective dilutions. Neurula stage embryos were fixed and examined at the by immunofluorescence following *in situ* hybridisation with antisense RNA probes. Retinoic acid (RA) activity was measured by chemiluminescence in neurula-stage embryos that had been injected with RAREZ reporter plasmid prior to Roundup exposure as described. For rescue experiments RAREZ-injected embryos were incubated with Roundup at 4000-fold dilution until the blastula stage, then exposed to the RA receptor antagonist Ro 41-5253 at 1.0 µM until assay of RA activity.

(ii) Two-cell *Xenopus* embryos were injected with 360 or 500 pg of glyphosate into one or both cells (producing intracellular concentrations of 8 – 12 µM) together with 10 ng of the visual marker Dextran Oregon Green. They were then incubated until sibling controls had reached the desired developmental stage, fixed, and examined visually or by immunofluorescence following *in situ* hybridisation as described above.

(iii) Fertilised chicken eggs were injected with 20 µL of 3500- or 4500-fold dilutions of Roundup Classic and incubated at 38℃ until fixation, *in situ* hybridisation and immunofluorescence examination as described for *Xenopus* embryos. Control embryos were treated similarly after injection of 20 µL of water.

Effects on neural crest markers, rhombomere formation and primary neuron differentiation: Compared with sibling controls, Roundup Classic at 5000-fold dilution impaired neural crest formation in 87% of *Xenopus* embryos (n = 30), seen as down-regulation of the neural crest marker *slug* and zinc finger transcription factor *krox-20* in the r3 rhombomere. Neuron formation was suppressed, as evidenced by decreased numbers of primary motor, inter- and sensory neurons in 83% of treated embryos. Similar effects occurred in 70 – 80% of embryos injected unilaterally with 500 pg glyphosate. On their injected side these displayed abolition of *slug* expression, reduced *krox-20* expression in r3 and r5, and decreased numbers of primary motor,

inter- and sensory neurons. The study authors considered the Roundup-exposed and glyphosate injected embryos to be equivalent (although not identical) phenotypes. They did not present any results obtained at the 360 pg/cell dose or the 1/4000 or 1/3000 dilutions.

Effects on the development of the head and dorsal midline: In 85% of 1:5000 Roundup-exposed neurula-stage *Xenopus* embryos, there was reduced expression of *shh* (a gene whose expression is responsible for resolving the brain and retina into two separate hemispheres) and *pax6* (responsible for eye formation). After incubation was prolonged to the tailbud stage, *ca* 90% of treated embryos displayed a decrease in anterior *shh* expression with concomitant microphthalmia, microcephaly, shortening of the anterior-posterior (A-P) axis and delayed migration of neural crest cells into the eyes, genital ridges and pharyngeal arches. Bilateral injection of 360 pg glyphosate also reduced *shh* expression and induced microphthalmia and microcephaly in the majority of treated embryos. In older (tadpole stage) embryos, Roundup exposure caused microphthalmia and a generalised reduction of cranial cartilage structures; most unilaterally-injected embryos showed these effects on the treated side, while bilateral injection caused cyclopia in 3/8 embryos. The study authors did not provide any data obtained at the 500 pg/cell dose or the 1/4000 or 1/3000 dilutions.

Effects on retinoic acid signalling: A highly significant ($p<0.0001$) dose-dependent increase in RA signalling activity occurred in Roundup-exposed *Xenopus* embryos at 4000- and 3000-fold dilutions. The magnitude of the effect was intermediate between the activity seen after addition of exogenous RA at 0.50 and 5.0 µM. However, there was no apparent response to Roundup at 1:5000, which Paganelli et al attribute to a lack of sensitivity of the RAREZ reporter plasmid. Assuming a linear response of the luminescence system, the study authors estimated that the endogenous concentration of RA in *Xenopus* embryos is *ca* 0.2 µM. The RA receptor antagonist Ro 41-5253 blocked the signalling increase mediated by 1:4000 Roundup, and prevented 1:5000 Roundup from inhibiting *shh* activity and causing microcephaly. No data were presented on the influence of Ro on RA signalling or embryo phenotype at other dilutions.

Effects in chicken embryos: Roundup caused concentration-dependent reduction in *pax6* expression and in the size of the optic vesicles, loss of the r3 and r5 domains and decrease in *shh* expression in midline cells, accompanied by microcephaly and loss of *shh* expression in the pre-chordal mesoderm.

Comment

The study authors suggest that the similarity between the phenotypes observed in Roundup-incubated and glyphosate-injected *Xenopus* embryos indicates that neural crest development is disrupted by the active constituent, rather than adjuvants present in the formulated product. Noting (a) similarities between the effects of Roundup and glyphosate with those of excess retinoic acid (RA) concentrations in *Xenopus*, mice and humans; (b) increased RA signalling levels in *Xenopus* embryos in response to Roundup; and (c) the effectiveness of the anti-retinoid Ro in preventing the developmental effects of Roundup in *Xenopus*, Paganelli et al hypothesise that glyphosate is a developmental toxin with a mode of action involving enhancement of RA signalling activity.

Given their belief that (d) glyphosate inhibits aromatase, a cytochrome P450 enzyme; and (e) retinoid activity is regulated by degradation of RA by CYP26, the study

authors further hypothesise that glyphosate increases RA signalling by inhibiting the activity of CYP26 responsible for maintaining normal RA distribution by specific territorial degradation.

Williams et al (2012) have noted that in this study

- The glyphosate solution was not pH-adjusted, and so the effects may be attributable to its acidic nature;
- The injection route of exposure was inappropriate and irrelevant to risk assessment; and
- The observations require further substantiation using appropriate methods before consideration in risk assessment.

Oliviera et al (2007): Adult drakes in breeding season (6/group) were gavaged with Roundup (360 g/L glyphosate, present as 480 g/L glyphosate isopropylamine salt; no other formulation constituents identified; Monsanto do Brasil Ltda, Sao Paulo, Brazil) in water at 5.0 or 100 mg/kg bw/d for 15 days. The study authors did not specify whether the dose levels applied to the active constituent, or the product. A control group received water only.

After the treatment period, the birds were anaesthetised and perfused intracardially with 2.5% gluteraldehyde. Fixed testes and epididymides (5/group) were then weighed, examined morphometrically and examined histochemically to investigate lysosomes and lipids within the epididymal region. Androgen receptor (AR) expression was studied by immunohistochemistry, with confirmation of antibody specificity by SDS-PAGE / Western blotting. Plasma testosterone and oestradiol concentrations were measured in three birds/group by RIA.

Results

Body and organ weights: There was no treatment-related effect on bodyweight. Relative testicular weights were depressed by *ca* 13% at both doses, but the difference from control was not statistically significant. Data on absolute testis weight were not presented.

Hormones: Plasma testosterone levels were reduced by *ca* 90% at both doses ($p<0.05$). A significant ($p<0.05$) *ca* 30% decline in plasma oestradiol occurred at 5.0 mg/kg, but there was no such effect at 100 mg/kg.

Tissue histology: Within the *testis*, Roundup at 5.0 and 100 mg/kg respectively induced slight but statistically significant ($p<0.05$ vs control) reductions in the volumetric proportion of seminiferous tubule epithelium (by 4 and 5%) and interstitial tissue (by 12 and 10%), together with 20 and 22% increases in the lumen volume ($p<0.05$). Spermatogenesis appeared to be normal, however.

Within the *epididymal region*, there were dose-related trends towards reduced volumetric proportions of proximal efferent ductules and connecting duct, together with increases in the proportion of rete testis, distal efferent ductules and connective tissue. These features attained statistical significance ($p<0.05$) at 100 mg/kg but not the low dose.

In the *proximal efferent ductules* of treated birds, qualitative morphological alterations (increased epithelial lipid content and epithelial vacuolisation caused by increased numbers of lysosomes) were found, together with increases of 11 and 7% in epithelial

height and 41 and 105% in lysosomal area at 5.0 and 100 mg/kg respectively (all $p<0.05$ vs control).

The morphology of the *epididymal duct* was also affected. Birds receiving 5 and 100 mg/kg, respectively, displayed significant ($p<0.05$) reductions of 28 and 49% in tubular diameter and increases of 23 and 34% in epithelial height. The epididymal ducts of treated birds presented collapsed and sometimes highly folded lumen, together with an increase in the basement membrane. By contrast, control birds presented wider and regular lumen and a slight basement membrane.

AR expression: At both doses, Roundup caused a major (but unquantified) decrease in AR expression within the Sertoli cell nuclei within the testis. However, the effect did not occur within the epididymal region. The specificity of the AR antibody used was confirmed.

Comment: This study is notable for the low numbers of birds used (especially for hormonal assay); the non-dose related depression of oestradiol concentration; and the lack of an experimental group treated with glyphosate alone, which prevented identification of the formulation constituent(s) causing the reported effects. The observed responses to treatment may have been associated with generalised physiological stress, rather than a specific effect on steroid hormone synthesis.

A4.5 Developmental and reproductive effects of a glyphosate-based herbicide in rats

Dallegrave et al (2003): Groups of 13 – 16 pregnant Wistar rats (90 days old, 200 – 280 g bw, bred at UFRGS, Porto Alegre, Brazil) received Roundup formulation (Lot BS 1096/98, Monsanto Brazil, containing 360 g/L glyphosate and 18% w/v POEA; no other components specified) by oral gavage at 500, 750 or 1000 mg glyphosate/kg bw/d[19] (and *ca* 250, 375 or 500 mg POEA/kg bw/d) (dose volume of 10 mL/kg in distilled water) from GD 6 – 15. Control rats received vehicle alone. Caesarean sections were performed on GD 21, and foetal bodyweight and the numbers of corpora lutea, implantation sites, live and dead foetuses and resorptions were recorded. Foetuses were examined for external malformations and skeletal alterations. However, there was no investigation of their internal organs.

Maternotoxicity: At 1000 mg/kg, there was 50% maternal mortality between GD 7 and 14, but the study authors did not describe any clinical signs or identify the cause of death. No mortality occurred at 0 – 750 mg/kg. There was no treatment-related effect on maternal water intake. The 750 mg/kg group displayed a consistent deficit of *ca* 2.0% in food intake over GD 3 – 21; this is not considered to be treatment-related because it was already present before dosing had commenced. Dams in the 1000 mg/kg group showed a deficit of up to *ca* 4.0% in food intake during the dosing period, maximising on GD 9 but reversing after cessation of treatment. This was accompanied by slight mean bodyweight loss between GD 6 and 9. Subsequent weight gain was similar to the other groups, except for a transient increase over GD 15 – 16. However, there were no statistically significant inter-group differences in food consumption or relative or total gestational bodyweight gain (which was 107, 85, 107 and 102 g at 0, 500, 750 and 1000 mg/kg). Also failing to attain significance was

[19] The doses are believed to have been based on glyphosate acid technical because Dallegrave et al stated that the dosing regimen was chosen by reference to a NOAEL for glyphosate of 1000 mg/kg bw/d for maternal and foetal effects in a developmental toxicity study in rats.

a dose-related trend towards increased relative liver weights (4.57, 4.73, 4.89 and 5.11% in the respective groups). Absolute organ weight data were not presented.

Litter parameters: At Caesarean section, there were 15, 15, 16 and 7 dams and 154, 148, 162 and 75 foetuses available for examination at 0, 500, 750 and 1000 mg/kg. There were no effects on implantation index, resorption rate, mean number of foetuses per dam or mean foetal bodyweight. Gravid uterus weight was not measured. The only remarkable litter parameter was an increase in male:female sex ratio to 1.5:1 at 1000 mg/kg, compared with 1.06:1, 1.01:1 and 0.94:1 in the control, 500 and 750 mg/kg groups. Nevertheless, the finding was not statistically significant (p=0.724, X^2 test) and there is no evidence that it arose from selective mortality of female foetuses *in utero.* Therefore, despite markedly reducing maternal survival at the high dose, the test formulation does not appear to have compromised foetal survival or growth.

Foetal development: There was no treatment-related effect on the incidence of external foetal malformations. However, as shown in the following table, an unequivocal treatment- and dose-related increase in skeletal alterations (all combined) occurred from 500 mg/kg upwards. These mainly involved ossification deficits suggestive of developmental delay but also included abnormalities such as absent ribs and caudal vertebrae, and wavy ribs. The most common individual alterations (incomplete skull ossification and enlarged fontanel) showed a dose-response relationship, but the incidences of some others were significantly ($p<0.05$) elevated at 750 and/or 500 mg/kg but not the high dose. It is not possible to exclude a relationship to treatment in these cases, because (a) no historical control or litter incidence data were presented, (b) the range of doses tested was very narrow, and (c) there were only half as many foetuses at 1000 mg/kg as in the remaining groups (which would reduce the chance of observing abnormalities).

Table 4.6: Percentage incidence of selected skeletal abnormalities in rat foetuses

Region or structure	Abnormality	Glyphosate Dose (mg/kg bw/d)			
		0	**500**	**750**	**1000**
Whole skeleton	All combined	15	33**	42**	57**
Skull, general	Incomplete ossification	10	29*	39*	56*
	Enlarged fontanel	1.9	26*	37*	53*
Interparietal	Bipartite	0.6	19*	4.9*	0.0
Supraoccipital	Bipartite	9.7	20*	1.2	0.0
	Incomplete ossification	3.2	0.0	1.2	13*
Maxilla	Short	0.6	0.7	0.0	1.3
Squama	Incomplete ossification	0.0	0.0	3.1*	2.7*
Caudal vertebrae	Absent	1.9	0.0	7.4*	15*
Ribs	Absent	1.3	2.7	3.1	4.0
	Incomplete ossification	1.9	2.0	5.6	4.0
	Wavy	0.6	2.0	4.9*	0.0
Sternebra	Incomplete ossification	1.9	14.9*	0.0	2.7
	Bipartite	3.9	14.2*	0.6	9.3
Limbs	Incomplete ossification	0.0	0.0	17.9*	1.3
Scapula	Incomplete ossification	0.6	3.4	1.2	4.0
Metacarpal bones	Incomplete ossification	1.3	1.4	0.6	2.7
Femur	Incomplete ossification	3.2	3.4	13*	0.0
Tibia / fibula	Incomplete ossification	2.6	2.7	12*	8.0
Metatarsal bones	Unossified	4.5	1.4	14*	11

Hind phalanges	Unossified	7.1	21*	22*	2.7
Ischium	Incomplete ossification	4.5	2.7	9.3	0.0
Pubis	Incomplete ossification	3.9	2.7	11*	0.0

*p<0.05 **p<0.001 vs control (X^2 test)

Conclusions

The NOEL for maternotoxicity was 750 mg glyphosate/kg bw/d, based on mortality and depression in food intake at the highest dose of 1000 mg/kg bw/d. There was no NOEL for effects on foetal development, due to increased incidences of skeletal abnormalities at and above the lowest dose of 500 mg glyphosate/kg bw/d.

Comment: Williams et al (2012) have criticised reporting deficiencies and anomalies in this paper, and also noted that foetuses were fixed in formalin and trypsin-digested prior to staining and skeletal examination instead of the standard method of alcohol fixation followed by maceration with potassium hydroxide. According to Williams, proteolysis could have digested peptide bonds in the bone matrix, creating areas that appeared to be incompletely ossified. Also deserving comment are the doses of POEA (*ca* 250, 375 and 500 mg/kg bw/d), which far exceed the maternal NOEL and LOEL of 15 and 100 mg/kg bw/d in rats (Holson, 1990). The mid and high doses are also greater than the foetal NOEL of 300 mg/kg bw/d[20].

Dallegrave et al (2007): Groups of 15 Wistar rats (90 days old, 250 – 350 g bw, bred at UFRGS, Porto Alegre, Brazil) received Roundup formulation (Monsanto Brazil, containing 360 g/L glyphosate and 18% w/v POEA; no other components specified) by oral gavage at 50, 150 or 450 mg glyphosate/kg bw/d (dose volume of 10 mL/kg in distilled water) throughout pregnancy and lactation. Control rats received vehicle alone. At delivery, litter size, the number of living and dead pups, birth weight and sex ratio were recorded. Offspring development was monitored by weekly evaluation of bodyweight and daily assessment of developmental landmarks including ear and eye opening, fur emergence, incisor eruption, testis descent, preputial separation and vaginal opening.

From each litter, one rat/sex was killed at puberty (PND 65 for males; first oestrus after PND 65 for females) and a further animal/sex was killed at adulthood (PND 140). Systemic toxicity was determined on the basis of the relative weights of the heart, lungs, liver, spleen, kidneys, adrenals and brain. Reproductive toxicity in males was evaluated as relative weight of the testis, epididymis, seminal vesicle with coagulating gland and prostate, together with spermatid and sperm numbers in the cauda epididymis, sperm morphology, testicular histology and blood testosterone concentration. In females, assessment of reproductive toxicity was limited to the relative weights of the uterus, oviducts and ovaries without histological examination.

Maternotoxicity and litter parameters: There were no maternal deaths or effects on relative bodyweight gain of dams during pregnancy or lactation. There were also no effects on litter parameters at birth, the survival and growth of pups during lactation or attainment of general developmental landmarks.

Female sexual characteristics: Vaginal patency was delayed by two to three days in the treated groups, which was statistically significant (p<0.05, ANOVA-Bonferroni

[20] In Holson (1990), rat dams gavaged with POEA over GD 6 – 15 showed clinical signs and decreased food consumption at 100 mg /kg bw/d, together with mortality and decreased bodyweight gain at 300 mg/kg. However, there were no foetal effects at 300 mg/kg bw/d, the highest dose administered (Williams et al, 2012).

test) vs controls. Latencies of 34.9, 37.6, 36.9 and 36.7 days were recorded at 0, 50, 150 and 450 mg/kg respectively. Nevertheless, the study authors did not consider the finding to be biologically significant because the latency period was "well within" historical control values (these were not cited, however). There was no effect on the weights of the reproductive organs.

Male sexual characteristics: Although there was no effect on attainment of testicular descent, preputial separation was advanced by one day in the 450 mg/kg group (see following table). Despite achieving statistical significance, this was not considered treatment-related because the latency was within the historical control range (not cited). Testis and accessory sex organ weights were not affected by treatment.

However, the numbers and morphology of sperms in the treated groups showed noteworthy displacements from control values, which the study authors considered were biologically significant. As shown in the table below, these comprised:

1. Statistically significant deficits of *ca* 25% in sperm numbers and daily sperm production at adulthood in the 50 and 450 mg/kg groups, although not at 150 mg/kg.
2. A statistically significant doubling in the proportion of abnormal sperm at puberty in the 50 mg/kg, with a non-significant increase at 450 mg/kg but little or no effect at the mid dose. At adulthood, all treated groups displayed a *ca* 1.5-fold elevation in abnormal sperm incidence relative to controls, which did not achieve significance (p=0.066, ANOVA). Furthermore, in the treated groups the proportion of sperm-producing tubules was depressed by *ca* 6 – 11% at puberty and 18 – 29% at adulthood.
3. Dose-related depression in serum testosterone levels, seen at all doses at puberty (significant at 450 mg/kg) but wholly or partially reversing by adulthood.
4. Histological abnormalities within the testis. At puberty, there were growth disorders and degeneration characterised by spermatid vacuolisation and a decrease in elongated spermatids at and above 150 mg/kg. At adulthood there was dose-related, intense tubular degeneration characterised by the absence of tubular lumen (see table).

Based on the above findings, the study authors considered that there was no NOEL for effects on the male reproductive system, and suggested that the test formulation was a probable endocrine disruptor. However, they acknowledged that the study had not elucidated a mechanism of action or identified which component of Roundup was causing the observed effects.

Table 4.7: Reproductive parameters (mean values) in male offspring

Parameter		Maternal glyphosate dose (mg/kg bw/d)			
		0	**50**	**150**	**450**
Age at preputial separation (d)		31.7	31.7	31.5	30.7*
Bodyweight at preputial separation (g)		73.0	68.1	72.2	70.7
Daily sperm production (x 10^6) (n=15)	**PND 140**	20.5	15.3*	19.7	14.7*
Sperm number (x 10^6) (n=15)	**PND 140**	345	251*	369	257*
Abnormal sperm (%) (n=15)	**PND 65**	8.6	16.7*	9.2	11.6
	PND 140	5.4	8.3	8.4	7.7
Tubules with spermatogenesis (%)	**PND 65**	84	77	79	75
(n=5)	**PND 140**	92	74	75	65
Blood testosterone concentration (ng/mL)	**PND 65**	5.2	4.0	3.2	1.5*

Parameter	Maternal glyphosate dose (mg/kg bw/d)			
	0	**50**	**150**	**450**
(n=15) PND 140	3.9	3.4	6.3	3.3
Testis: spermatid vacuolisation & decrease in elongated spermatids (incidence at PND 65)	NS	NS	4/5	4/5
Testis: tubular degeneration (incidence at PND 140)	NS	3/5	4/5	4/5

*p<0.05 vs control, ANOVA – Bonferroni test
NS = Not stated

Comment

Interpretation of the results is hindered by the lack of historical control data, which may have defined effect levels and clarified whether there were genuine treatment-related effects on variables that did not show dose-response relationships. These include daily sperm production, sperm numbers in the cauda epididymis and the proportion of abnormal sperms, which showed the least displacement at 150 mg/kg. The reviewing toxicologist considers that the reporting of histological findings in the testis was insufficiently detailed, as it lacked descriptive detail, severity gradings and control data. The study would also have been strengthened by histological examination of the female reproductive organs.

In an independent assessment of this study, Williams et al (2012) have remarked that:

- In the 450 mg/kg bw/d group, the age at preputial separation was within the physiological range for rats;
- Hastening of puberty would be not be expected, given that the 450 mg/kg group had the lowest mean circulating testosterone level on PND 65;
- The increased percentage of abnormal sperm at 50 mg/kg bw/d may be a random finding, given the lack of effects at higher doses;
- Dallegrave et al's reporting of the testicular histology was deficient and the abnormalities described may be a tissue processing artefact, rather than an effect of treatment;
- Testicular abnormalities have not been reported in offspring in reproduction studies with glyphosate, all of which involved much greater glyphosate exposures.

Conclusions

In the absence of any apparent maternotoxicity, the NOEL in dams was 450 mg glyphosate/kg bw/d. The study did not demonstrate treatment-related effects in female offspring at up to and including the highest dose of 450 mg glyphosate/kg bw/d. The study is considered to be insufficiently reliable enough to demonstrate whether there were treatment-related effects in male offspring.

Romano et al (2010): The test compound in this study was Roundup Transorb (Monsanto Co, St Louis, MO, USA / Monsanto of Brazil Ltda, Sao Paulo, Brazil; containing glyphosate isopropylamine salt 648 g/L equivalent to 480 g/L glyphosate, with 594 g/L of unidentified "inert ingredients"). The formulation was diluted in water to yield a dosage volume of 0.25 mL/100 g bw, and administered PO by gavage to newly weaned male Wistar rats (16 – 18/group) from PND 23 – 53 at 5.0, 50 or 250 mg/kg bw/d. A control group received vehicle alone. The study authors described their test compound as "glyphosate-Roundup Transorb", so it is ambiguous whether they were referring to the active or product. However, given that their choice of doses

was based on a NOEL of 50 mg/kg bw/d *for glyphosate* in another study, it will be assumed that the doses are equivalent to 5.0, 50 or 250 mg active/kg bw/d.

Pups were weighed daily throughout the treatment period and examined to determine the age of puberty (balano-preputial separation) from PND 33 onwards. At termination on PND 53, serum was collected via cardiac puncture for measurement of testosterone, oestradiol and corticosterone concentrations. The testes and adrenal glands were weighed and processed for histological examination. Quantitative morphometry of the seminiferous tubules was then performed to examine for disturbance of spermatogenesis. However, spermatozoa were not examined or quantified.

There were no treatment-related effects on bodyweight throughout the dosing period, including puberty ($p>0.05$). However, attainment of puberty was delayed by *ca* 1.0 and 1.5 days at 50 and 250 mg/kg respectively ($p<0.01$ and <0.001 vs control). As shown in the following table, relative testicular weight increased dose-relatedly by up to *ca* 9%, attaining statistical significance at 250 mg/kg. At this same dose, there was also a significant, 29% increase in relative adrenal weight. Absolute organ and terminal body weights were not provided.

Serum testosterone concentrations were depressed by 30%, 45% and 50% at 5, 50 and 250 mg/kg bw/d. Histologically, this finding was correlated with decreased numbers of germ cells, seen as a dose-related reduction in the height of the seminiferous tubule germinal epithelium and increased diameter of the lumen. Displacements from control were statistically significant at all doses (see table below). By contrast, serum corticosterone and oestradiol concentrations, adrenal morphology and the overall diameter of the seminiferous tubules were not affected.

Table 4.8: Treatment-related effects in rats

Variable examined	**Dose (mg/kg bw/d)**			
	0	**5**	**50**	**250**
Mean testicular weight (mg/100 g bw)	531	539	553	580*
Mean adrenal weight (mg/100 g/bw)	11.3	12.8	12.3	14.6*
Serum testosterone concentration (ng/dL)	155	109**	85***	77***
Seminiferous tubule: Germinal epithelium height (µM)	86	72**	69**	65**
Lumen diameter (µM)	94	117**	114**	130**

*p<0.05 **p<0.001 vs control

Comment

The study was performed before the publication of the EPA OPPTS Test Guideline 890.1500 for investigating pubertal development in male rats[21], but the treatment period (PND 23 – 53) was in line with the Guideline-specified protocol. However, the study was not Guideline-compliant in numerous other aspects of its design and reporting. In particular, there were no bodyweight data except for the mean values at preputial separation. It is therefore impossible to verify independently that inter-group variation in bodyweight and/or bodyweight gain did not influence the timing of puberty, or other parameters. It is also unclear whether the experimenters ensured that litter mates were not allocated to the same experimental group, as required by the Guideline.

[21] Endocrine disruptor screening program Test Guideline OPPTS 890.1500: Pubertal development and thyroid function in intact juvenile/peripubertal male rats. EPA 740-C-09-004, October 2009.

Furthermore, Williams et al (2012) have questioned the reliability of the preputial separation data and morphometric analysis of testis pathology, claiming that the latter was affected by tissue fixation artefacts and confounded by variation in the maturity of seminiferous tubules.

Conclusions

The study is considered to be insufficiently reliable to demonstrate whether there were treatment-related effects in the experimental model used.

Romano et al (2012): Roundup Transorb (see Romano et al, 2010) was administered to pregnant Wistar rats PO by gavage at a dose equivalent to 50 mg glyphosate/kg bw/d from GD 18 to PND 5. The test compound was diluted in water and given at a dose volume of 2.5 mL/kg bw. A control group (size unspecified) received water alone. On PND 4, litters were culled to eight pups/dam and then maintained until weaning at PND 21. Their bodyweight was recorded on PND 21, 30, 40 and 60. Throughout the post-weaning period, male offspring were evaluated for preputial separation, indicating attainment of puberty.

Preference test: On PND 60, subgroups of five male offspring from treated and control dams alternately underwent a sexual preference test, in which they were placed individually on a circular stage with one male and one female stimulus rat, housed in separate cages on opposite sides of the apparatus. The stage was divided into neutral, male and female areas, with the male and female areas divided into seven zones. Stimulus males were gonad-intact and sexually mature, whereas the stimulus females had been ovariectomised and brought into oestrus with oestradiol (50 µg/kg SC at -54 hours) and progesterone (2.0 mg/kg SC at -6 hours). After a five-minute adaptation interval, there was a 20-minute observation period during which the test males' stay times in the two zones nearest the stimulus males and females were recorded. Preference scores were calculated by subtracting the total time spent in the male zones from the time spent in the female zones. Following the preference trial, the test males were not subjected to other experiments.

Mating behaviour: Four males from treated and control dams were scored for the numbers of mounts, attempted mounts, intromissions and ejaculations over a 40-minute interval when placed individually with an oestrus-induced female rat. The time to first ejaculation and ejaculatory intervals were also recorded.

Reproductive tract: On PND 60, the testes, epididymides (caput, corpus and cauda) and seminal vesicles were weighed, sperm counts were performed, and the histology and morphometry of the seminiferous epithelium examined by light microscopy.

Other parameters: Serum concentrations of testosterone and oestradiol were measured by RIA, and FSH and LH concentrations were measured using chemiluminescence immunoassay. Pituitary mRNA and protein levels of β-LH, β-FSH and GH were analysed by real-time PCR (for mRNA) and SDS-PAGE followed by nitrocellulose membrane hybridisation / antibody detection (for proteins).

Results

Maternal observations: No information was provided on the survival, appearance, behaviour or bodyweight of dams during or after the dosing period. It is therefore unknown whether any maternotoxicity occurred.

Growth of offspring and attainment of puberty: The study authors did not present data on bodyweight or pituitary GH levels, but claimed that neither was affected by treatment. In males from Roundup-treated dams, however, age and bodyweight at preputial separation were decreased by about two days (mean of 45 vs 47 days; $p<0.05$) and 30 g (mean of 215 vs 245 g; $p<0.05$).

Preference test: As shown in the table below, male rats from the Roundup-treated dams spent significantly longer in close proximity to female stimulus animals, and had a significantly higher preference score.

Table 4.9: Results of sexual preference test

Parameter	Time (sec)	
	Control	Roundup
Mean total time in male area	431	312
Mean total time in female area	502	625**
Mean partner preference score	71	313**

**p<0.01 vs control (Student's t-test) N = 5/group

Mating behaviour: Based on the interquartile ranges, the study authors claimed a significant increase in mounting, intromission and ejaculatory latency for males from Roundup-treated dams. The remaining parameters did not differ significantly between the groups.

Table 4.10: Results of mating behaviour evaluation

Parameter	Time (min)	
	Control	Roundup
Latency for the first mount^	0.6 – 1.0	5.2 – 7.0*
Latency for the first intromission^	0.6 – 1.0	5.2 – 7.0*
Latency for the first ejaculation^	1.0 – 1.7	5.5 – 7.0*

^Data are interquartile range (25 – 75%) N = 4/group
*p<0.05 vs control (Mann-Whitney U-test)

Reproductive tract: There were no effects on the relative weights of the testes or undrained seminal vesicles on PND 60. However, the relative weight of drained seminal vesicles was 10% higher in the Roundup group, suggesting a lower fluid volume. The corpus and cauda segments of the epididymis were slightly but significantly heavier in the Roundup group than controls. Compared with controls, sperm production was approximately twice as high in rats from Roundup-treated dams (see table below), and sperm reserves in the caput + corpus were increased by 50%. Sperm transit time through the cauda was reduced by *ca* 1/3rd. In the absence of any significant difference in the diameter of the seminiferous tubules, the Roundup group displayed a minor but statistically significant increase in epithelial height and decrease in luminal diameter.

Table 4.11: Findings in the reproductive system of male rats

Parameter		Control	Roundup
Total sperm production (X 10^6/testis)		52	99*
Total sperm production (X 10^6/g testis)		35	71*
Daily sperm production (X 10^6/testis)		8.5	16*
Daily sperm production (X 10^6/g testis)		5.7	12*
Sperm reserve, caput + corpus (X 10^6)		14	21*
Sperm transit time through cauda (days)		6.3	4.0*
Seminiferous	Tubular diameter (µm)	467	451

epithelium	Epithelial height (µm)	92	98*
	Luminal diameter (µm)	257	239*
Seminal vesicle	Weight, undrained (mg/100 g bw)	160	155
	Weight, drained (mg/100 g bw)	100	110*
Epididymis	Weight, corpus (mg/100 g bw)	10	13*
	Weight, cauda (mg/100 g bw)	36	43*

*p<0.05 vs control (Student's t-test) N = 8/group

Other parameters: In males from Roundup-treated dams, serum testosterone and oestradiol concentrations were approximately twice as high as in controls (see following table). Pituitary LH and FSH mRNA levels were very slightly but significantly increased by Roundup treatment. However, although there were concomitant increases of *ca* 70% in pituitary LH protein and serum LH levels, there was no treatment-related effect on FSH levels in the pituitary or serum.

Table 4.12: Hormonal levels in the serum and pituitary

Parameter	Control	Roundup
Serum testosterone conc. (ng/dL) (N = 12)	60	140**
Serum oestradiol conc. (pg/mL) (N = 12)	1.4	2.8**
Pituitary LH mRNA content (AU) (N = 8)	1.00	1.02*
Pituitary LH protein content (AU) (N = 8)	1.1	1.9**
Serum LH conc. (pg/mL) (N = 8)	70	120*
Pituitary FSH mRNA content (AU) (N = 8)	1.00	1.02*

*p<0.05 **p<0.01 vs control (Student's t-test)

Conclusions

The study authors interpreted their findings as indicating that maternal glyphosate exposure during the perinatal period caused hypersecretion of androgens in the male offspring, combined with hastening of puberty, increased gonadal activity and sperm production, greater predilection for the company of female rats and increased libido (the latter notwithstanding the statistically significant increase in the *delay* before copulation). The authors acknowledged that their findings contradicted the depression in serum testosterone level and sperm production and reduced height of the seminiferous epithelium observed by Romano et al (2010) and Dallegrave et al (2007) (see above). However, they attributed the discrepancies in experimental outcome to differences in timing of exposure, which occurred over GD18 to PND 5 in this study but extended through gestation to the end of lactation (PND 21) in Dallegrave et al (2007) and was from PND 23 to 53 in Romano et al (2010).

Comment

Numerous aspects of the design of this study and its findings deserve comment.

- Although the study authors attribute their findings to glyphosate, dams were treated with a commercial formulation containing 594 g/L of unidentified "inert ingredients". Offspring may consequently have been exposed to these formulation adjuvants *in utero* or via maternal milk and it is possible that they influenced the experimental outcome, either directly or by interaction with the active constituent. The study did not control for the presence of adjuvants.

- Since no observations on the dams were presented, it is unknown whether maternotoxicity (including effects on maternal nursing behaviour) occurred. The study authors appear not to have considered the possibility that at least some experimental findings in offspring arose from effects on the mothers.
- The study authors did not state when serum and pituitary hormone parameters were measured.
- Rats that underwent the sexual preference test were not used for other experiments, but no information was provided on whether those undergoing evaluation of mating behaviour were also subjected to hormone assays and/or reproductive tract histology. Either of these end-points could have been affected by sexual activity.
- In a mating evaluation, one would expect relatively large variation in the behaviour of individual males, especially given that the outcome would be partially dependent on the behaviour of the partnering females. However, the group sizes were very small (N = 4). No group mean values were provided; data were reported as interquartile ranges (25 – 75%). In a set of four observations, there would be only one data point per quartile. Therefore, because they were based on so few observations, it is open to question whether the apparent increases in mounting, intromission and ejaculation latency time were biologically significant, even though statistical significance was attained.
- In an extensive critique of this study, DeSesso and Williams (2012) point out that surfactants inhibit the enzyme aromatase, which is responsible for conversion of circulating testosterone to oestradiol. Surfactants, if present in the test formulation, could therefore have disrupted the expression and function of endocrine hormones in the dams and/or offspring.
- The study authors did not identify from which dams/litters the evaluated males had originated. DeSesso and Williams question whether the study was controlled for litter effects, adding that because litter mates are more similar to each other than offspring from separate litters, the observed inter-group differences may be due to animals being derived from the same limited number of litters rather than a true effect of treatment.
- DeSesso and Williams note the lack of evidence that precautions were taken to prevent the sexual preference test being confounded by environmental cues including auditory and visual stimuli, odours and pheromones.
- These authors also observe major differences in the control values for attainment of puberty, serum testosterone and oestradiol concentrations and seminiferous tubule morphometry when comparing Romano's 2010 and 2012 studies. The magnitude of these differences exceeds the size of the treatment-related changes within each study.
- Romano et al (2010; see above) report that preputial separation in controls occurred at means of *ca* 37 days and 146 g bw, compared with 47 days and 245 g bw in their 2012 paper. Mean values from test animals in 2012 (45 days and 215 g bw) lie within this range, and also within the range specified for control Wistar rats in US EPA TG 890.1500 (40 – 46 days and 177 – 241

g bw)[22]. By contrast, mean values from controls in both studies lie *outside* the EPA's Guideline ranges (DeSesso and Williams, 2012).

A4.6 Reproductive effects of glyphosate in male rabbits

Yousef et al (1995): Following an initial six-week observation period, male NZW rabbits (4/group, 8 months old, mean initial bodyweight 2863 g) were given oral doses of glyphosate (from Monsanto, USA) in gelatin capsules for six weeks at 1% or 10% of the LD_{50}. The study authors did not explicitly identify the dosing interval or specify the doses in terms of mg/kg bw. The rabbits were then held without treatment for a further six weeks to study reversibility of effects. The animals were weighed weekly in the morning before access to feed and water. Semen was collected weekly throughout the study using a teaser doe and artificial vagina, with ejaculate volume being recorded after removal of the gel mass. Semen osmolality, fructose concentration and methylene blue reduction time was measured together with sperm concentration and assessment of live, dead and abnormal spermatozoa.

Results

No information was provided on survival of the test and control animals, but for reasons unknown, one rabbit was removed from the control, low and high dose groups during the recovery period. Other than stating that most treated animals showed indications of reduced libido (especially at the high dose), the study authors did not comment on clinical signs. Control mean bodyweight increased by *ca* 2 and 3% respectively during the treatment and recovery periods. By contrast, the low and high dose groups lost weight during treatment, with weight loss being greatest at the low dose (see following table). During recovery, there was little bodyweight change at the low dose, whereas the high dose group showed a bodyweight gain of *ca* 8%.

Table 4.13: Bodyweight (g) of rabbits over the experimental period

Time period	N	Control	GLY 1/100th LD50	GLY 1/10th LD50
Pre-treatment	4	2944	2979	3173*
Treatment	4	3008	2811*	3125
Bw change over treatment^		+64	-168	-48
Recovery	3	3108	2816*	3368*
Bw change over recovery^		+100	+5	+243

^Calculated by evaluator *p<0.05 vs control

Treated rabbits displayed a partially reversible, non-dose related *ca* 25% reduction in semen volume during the treatment period, accompanied by a reversible 3-fold increase in the percentage of dead sperm and partially reversible, dose-related depression in initial semen fructose concentration and prolongation in methylene blue reduction time (see next table). According to the study authors, fructose formation by the accessory glands is dependent on testosterone production by the testis; hence, decreased semen fructose suggested a corresponding decline in testosterone secretion. Yousef et al considered that prolonged MBRT could reflect deficits in nutrition status, viability, activity and oxygen consumption by sperm from treated rabbits.

Interpretation of sperm concentration data is confounded by a progressive doubling in the control group between the pre-treatment and recovery periods. By contrast, sperm concentration in the high dose group remained constant during treatment, but declined

[22] Endocrine disruptor screening program Test Guideline OPPTS 890.1500: Pubertal development and thyroid function in intact juvenile/peripubertal male rats. EPA 740-C-09-004, October 2009.

by *ca* 8% at the low dose. In both treated groups, sperm concentration then rose by *ca* 1.8-fold during recovery. The percentage of abnormal sperm became significantly ($p<0.05$) elevated in the treated groups during the dosing and recovery periods, but again, interpretation is confounded by a two-fold increase in abnormal sperm occurring in controls (mainly) during recovery. The most common types of abnormalities were claimed to be coiled or double tail and tapering or small head. Semen osmolality in treated rabbits changed little during the dosing period, but became statistically significantly lower than in controls. This was caused by increased osmolality in the control group, rather than any effect of treatment.

Table 4.14: Semen characteristics of rabbits. Values are overall means over 6 weeks before, during and after treatment. [n = 4 before and during treatment and n = 3 during recovery]

Parameter	Time period	Control	GLY 1/100th LD50	GLY 1/10th LD50
Semen volume (mL)	P	0.88	0.83	0.88
	T	0.83	0.60*	0.62*
	R	0.82	0.68*	0.73*
Sperm conc. (X 10^6/mL)	P	264	265	262
	T	413	242*	262*
	R	596	473*	467*
Abnormal sperm (%)	P	9.4	9.7	10.3
	T	12.5	21.9*	22.6*
	R	20.4	25.7*	24.1*
Dead sperm (%)	P	6.6	6.4	6.5
	T	8.9	19.5*	21.4*
	R	4.1	6.2*	7.5*
Methylene Blue Reduction Time (min)	P	5.07	5.22	5.07
	T	3.53	6.54*	7.26*
	R	3.48	5.0*	5.29*
Initial fructose conc. (mg/100 mL)	P	337	324	336
	T	359	281*	267*
	R	312	298	297
Semen osmolality (units unstated)	P	248	255	253
	T	283	252*	261*
	R	278	284	278

P = Pre-treatment T = Treatment period R = Recovery *$p<0.05$ vs control

Comment

The study has significant shortcomings in its design and reporting of the experimental methods and results. The dosing interval and administered doses of glyphosate are unknown, and the authors did not explain how the reference LD50 value was derived. Although glyphosate treatment does appear to have caused decreases in ejaculate volume, sperm viability and sperm activity (the latter possibly resulting from depression in semen fructose concentration), the causal mechanism is unidentifiable. It is uncertain whether the results were obtained in the presence of systemic toxicity, as bodyweight loss during treatment was three-fold more severe at the low dose than the high dose. Any effects on semen osmolality, sperm concentration and sperm morphology are uninterpretable due to major, unexplained variation over time within the control group. The small size of the experimental and control groups may have contributed to the experimental outcome.

Furthermore, Williams et al (2000) have observed that:

- The rabbits used in this study were small for their age, bringing into question their health status and reproductive maturity;
- The proper method of semen collection was not used. Multiple ejaculates were not pooled to decrease the inter- and intra animal variability in sperm number and concentration;
- Sperm concentration data from treated and control rabbits were within the normal range in mature NZW rabbits; and
- It is unclear whether control animals were subjected to sham handling and dosing procedures, raising questions of indirect non-treatment related effects given the sensitivity of rabbits to stress.

Based on these deficiencies, the data from this study cannot be used to support any meaningful conclusions.

A4.7 Dermal carcinogenicity of a glyphosate-based herbicide in mice

George et al (2010): Carcinogenicity bioassay: The biological activity of Roundup Original* (a commercial formulation containing 360 g/L glyphosate acid equivalent as the isopropylamine salt, with 15% POEA; no other components identified; manufactured by Monsanto Co., St Louis, MO USA) was tested in a mouse two-stage initiation / promotion model of dermal carcinogenesis. Eight groups of 20 male Swiss mice (12 – 15 g initial bodyweight; from the Indian Institute of Toxicology Research breeding colony) were treated according to the following scheme:

Group	Treatment protocol
1	Nil
2	Roundup*, 25 mg/kg bw, 3X/wk for 32 wk
3	DMBA, 52 µg/mouse, single dose then TPA, 5µg/mouse, 3X/wk for 32 wk
4	Roundup, 25 mg/kg bw, single dose then TPA, 5µg/mouse, 3X/wk for 32 wk
5	Roundup, 25 mg/kg bw, 3X/week for 3 wk then TPA, 5µg/mouse, 3X/wk for 32 wk
6	DMBA, 52 µg/mouse, single dose
7	TPA, 5µg/mouse, 3X/wk for 32 wk
8	DMBA 52 µg/mouse single dose then Roundup, 25 mg/kg bw, 3X/wk for 32 wk

*The study authors include Roundup Original, but not pure glyphosate, in the list of experimental materials. They state that mice were treated with "glyphosate 25 mg/kg bw". It is unclear whether they mean "Roundup at 25 mg/kg bw" [in which case the dose of glyphosate would be 9 mg/kg bw], or "sufficient Roundup to deliver a glyphosate dose of 25 mg/kg bw". I have assumed the former, and use the name "Roundup" to preserve the distinction between the active constituent and commercial formulations bearing this trade name.

DMBA = 7,12-dimethyl benz[a]anthracene
TPA = 12-*O*-tetradecanoyl-phorbol13-acetate

The initiator (DMBA), promoter (TPA) and Roundup formulation were applied to the clipped intact dorsal skin. According to the study authors, "Vehicle for glyphosate, DMBA and TPA were 50% ethanol and acetone, respectively" [sic]. Animals were weighed and examined weekly for the presence of tumours. All mice were sacrificed after 32 weeks of treatment.

Proteomic study: Groups of four male mice (which had not been used for the carcinogenicity bioassay) were treated dermally once with Roundup (50 mg/kg bw), DMBA (104 µg/mouse) or TPA (10 µg/mouse). The study authors did not state whether vehicles were used. A further four untreated animals served as controls. At 24 hours post-treatment, mice were sacrificed and skin samples from the treatment sites were excised, homogenised, lysed, sonicated, centrifuged and pooled for each respective group. Proteins in the supernatants were then separated by two-dimensional gel electrophoresis (2-DE), with the first dimension on immobilised pH gradient strips (pH 3 – 10) and the second dimension on polyacrylamide gel. Analysis was performed in triplicate. Protein expression levels were measured using PDQuest software, and protein spots that varied > two-fold from control were identified by matrix-assisted laser desorption / ionisation time-of-flight and liquid chromatography / mass spectrometry. The identity of some proteins was confirmed by immunoblotting.

Results

Carcinogenicity bioassay: All mice survived until scheduled termination. All 20 positive controls (Group 3 animals treated with DMBA and TPA) developed skin tumours (squamous cell papillomas), with some animals bearing multiple tumours (see Table). Skin tumours also developed on eight / 20 mice receiving DMBA and Roundup. Compared with TPA, Roundup induced the formation of fewer (by 85%), smaller tumours, which first appeared after a more prolonged (by 2.5-fold) treatment period. There was no comment on whether the tumours were preceded or accompanied by dermal irritation or other visible abnormalities at test sites. No dermal tumours were observed on mice from Groups 2, 4, 5, 6 or 7. Therefore, Roundup behaved as a tumour promoter in this experimental model, but not as an initiator or complete carcinogen.

Table 4.15: Tumour formation on the skin of treated and control mice

Group	Treatment	Incidence of TBM^	Days until 1st tumour	% of TBM^	Total no. of tumours	Mean no. tumours / mouse	Mean tumour vol (mm^3)/TBM^
1	None	0 / 20	NA	0	0	0	NA
3	DMBA + TPA	20 / 20*	52	100	156	7.8	96.4
8	DMBA + Roundup	8 / 20*	130	40	23	2.8	26.2

^TBM = Tumour bearing mice NA = Not applicable
*$p<0.05$ vs untreated controls (ANOVA)

Proteomic study: As revealed by 2-DE, single doses of Roundup, TPA or DMBA caused a >two-fold increase or decrease in the expression of 22 proteins. Expression levels of 13 of these proteins were said to be affected similarly by Roundup and TPA, but quantitative data were provided for only nine of these (see Table). DMBA up-regulated four of this sub-set of proteins similarly to Roundup and TPA, but had little or no effect on the expression of superoxide dismutase 1 (see Table). Use of the Western blotting technique confirmed that Roundup and TPA both up-regulated calcyclin and calgranulin-B by *ca* three- and four-fold, respectively, and down-regulated superoxide dismutase by about ten-fold (all $p<0.05$ vs control). Western blotting also demonstrated that DMBA did not influence the expression levels of these particular proteins.

Table 4.16: Expression levels of skin proteins in mice

Protein	Difference from untreated control		
	Roundup	TPA	DMBA
Translation elongation factor eEF1A1	+2.80	+2.79	+2.67
Carbonic anhydrase III	+1.62	+3.72	+2.81
Calcyclin	+2.48*	+2.20*	ND
Annexin II	+2.38	+1.72	ND
Fab fragment of anti-VEGF antibody	+3.64	+3.69	+5.80
Peroxyredoxin-2	+2.73	+2.74	+2.20
Superoxide dismutase 1	-4.97*	-4.56*	+1.16
Stefin A3	+2.29	+1.49	ND
Calgranulin-B (two "spots" corresponding to the same protein)	+9.52*	+7.61*	ND
	+9.34*	+7.43*	ND

*p<0.05 vs control ND = Not detected using 2-DE

Conclusions

The study authors concluded that glyphosate is a tumour promoter in mouse skin which, based on the similarities in protein expression profile, has a mechanism similar to TPA. They noted that several of the proteins whose activity levels were up-regulated have biologically significant roles in cell proliferation[23], while suggesting that down-regulation of superoxide dismutase (which protects cells against reactive oxygen intermediates) could potentiate tumour formation.

Comment

In the reviewing toxicologist's opinion, the carcinogenicity bioassay was not controlled adequately. The test compound was a mixture containing glyphosate, POEA and possibly other adjuvants, and yet no animals were treated with glyphosate, POEA or other formulation constituents in isolation. Therefore, the study could not identify which formulation constituent(s) promoted the growth of tumours in Group 8, show that tumour promotion was caused by any single chemical, or exclude the possibility that promoting activity arose from an interaction between two or more formulation components.

The study reporting would have been strengthened if the authors had commented on whether Roundup Original caused irritation or other effects on the skin where it was applied. This would have been of particular interest because POEA is a severe dermal irritant (Birch, 1977), consistent with the properties of surfactants in general, which interact with and solubilise lipid components of the skin and mucous membranes (Williams et al, 2000). The presence or absence of dermal responses such as inflammation, de-fatting, cell proliferation, scabbing, scarring or fissuring could have assisted in identifying the mechanism(s) by which Roundup promoted the formation of tumours. In this context, it is notable that POEA is not a mutagen (Stegeman and Li, 1990; Williams et al, 2000).

[23] According to the study authors, **Translation eF1A1** is responsible for binding aminoacyl-tRNA to ribosomes during polypeptide synthesis and its increased expression is directly proportional to cellular proliferation, oncogenic transformation, apoptosis and delayed cell senescence; **Carbonic anhydrase III** is involved in the cellular response to oxidative stress; **VEGF** is involved in angiogenesis (a pre-requisite for neoplastic growth); **Stefin A3** plays a role in skin growth and its induction by TPA leads to keratinocyte differentiation and proliferation; **Annexin II** is up-regulated in several human cancers; **Peroxyredoxin-2** is over-expressed in some cancers; and **Calcyclin** and **Calgranulin-B** are implicated in cell cycle progression, differentiation, cancer development and metastasis.

Another point deserving comment is that the proteomic analysis was carried out only at 24 hours after a single application of DMBA, Roundup or TPA. This is fundamentally different from the carcinogenicity bioassay, which involved repeated dosing over 32 weeks after DMBA application. No analysis was performed on skin from test sites during or at the end of the treatment period, on the tumours themselves, or on skin that had been treated with both DMBA *and* Roundup or TPA. The study did not demonstrate that the changes in protein expression observed after one dose of DMBA, TPA or Roundup were sustained throughout the experimental period, were a toxicological endpoint rather than homeostatic regulation, or were causally associated with the eventual development of tumours. Furthermore, the study could not detect changes in the expression of additional proteins after repeated treatment. Consequently, it is uncertain that the promoting activity that the study authors attributed to glyphosate is mechanistically similar to that of TPA.

Overall, this study has shown that Roundup Original is a tumour promoter on mouse skin, its activity is weaker than that of the positive control, TPA, and is dependent on prior induction with the initiator DMBA. The causative agent(s) and its (or their) mode of action remain unidentified. Given that Roundup Original is not a complete carcinogen, it is unlikely to pose a carcinogenic hazard for persons exposed dermally.

A4.8 Epidemiological Study

Eriksson et al (2008): This was a population-based case-control study of exposure to pesticides as a risk factor for non-Hodgkin lymphoma (NHL), consisting of 910 cases and 1016 controls. The subjects were men and women aged 18 – 74 years living in Sweden, diagnosed with NHL between December 1999 and April 2002. All cases were diagnosed and classified histopathalogically according to WHO criteria. Controls were selected from the national population registry.

Exposure assessment was performed by a questionnaire which included work history, exposure to pesticides, organic solvents and several other (unidentified) chemicals. For dose-response analysis of pesticides, information was collected on the number of years, days per year and hours per day of exposure. The questionnaire also included smoking habit, medications, leisure activities and residential proximity to industrial installations, but data on these variables were not included in the review. Supplementary phone interviews were conducted if necessary. All exposures of less than a full day, or occurring during the same calendar year as the diagnosis or one year prior, were disregarded.

Data were analysed by unconditional logistic regression (univariate and multivariate) adjusted for age, sex and year of diagnosis or enrolment. In the univariate analysis, different pesticides were analysed separately, and the unexposed category consisted of subjects who were not exposed to any of the included pesticides. All controls were used in the analyses of NHL subgroups. In the dose-response calculations made for agents with at least 20 exposed subjects, the median number of days of exposure among controls was used as a cut-off. Latency period calculations and multivariate analyses (performed because most pesticide exposures involved more than one chemical) included agents with statistically significantly increased ORs, or with an OR >1.50 and at least 10 exposed subjects.

Results

Univariate analysis adjusted for age, sex and year of diagnosis or enrolment revealed a significant association between NHL and exposure to glyphosate (29 cases and 18 controls; OR = 2.02; 95% CI = 1.10 – 3.71), exposure to glyphosate with a latency of >10 years before diagnosis (unstated no. of cases and controls; OR = 2.26; 95% CI = 1.16 – 4.40) and exposure to glyphosate for >10 days (17 cases and 9 controls; OR = 2.36; 95% CI = 1.04 – 5.37). However, NHL was not associated with exposure to glyphosate with a latency of 1 – 10 years before diagnosis (unstated no. of cases and controls; OR = 1.11; 95% CI = 0.24 – 5.08) or exposure to glyphosate for <10 days (12 cases and 9 controls; OR = 1.69; 95% CI = 0.70 – 4.07). Multivariate analysis adjusting for exposure to other chemicals yielded a low and statistically non-significant risk estimate for glyphosate (OR = 1.51; 95% CI = 0.77 – 2.94).

When the different sub-types of NHL were analysed separately, exposure to glyphosate was associated with a significantly enhanced risk of *small lymphocytic lymphoma / chronic lymphocytic leukaemia* (195 cases; OR = 3.35; 95% CI = 1.42 – 7.89) and *unspecified NHL* (38 cases; OR = 5.63; 95% CI = 1.44 – 22.0). Odds ratios for other types of lymphoma were not statistically significant.

Comment

The same research group have published a previous (Hardell et al, 2002) epidemiology study on the association between pesticide exposure and NHL, in which univariate analysis found a significant association with glyphosate (OR = 3.04; 95% CI = 1.08 – 8.52) based on 8 cases and 8 controls. Noting the small sample size and the broad CI, the Australian DoHA (2005) concluded that strength of association was questionable, and it was equivocal whether glyphosate was indeed a risk factor for NHL.

The current follow-up study improves on its predecessor in several respects, as it was based on a larger population (910 vs 515 cases), had larger sample sizes, included both men and women, and collected exposure data from living individuals only.[24] The follow-up would therefore have increased statistical power and diminished recall bias. Compared with the 2002 study, the risk estimate was lower (OR of 2.02 vs 3.04) but the association between glyphosate exposure and NHL was strengthened, as evidenced by the narrower 95% CI (1.10 – 3.71 vs 1.08 – 8.52). However, the 2008 and 2002 studies failed to demonstrate associations by multivariate analysis, which yielded ORs of only 1.51 and 1.85, with 95% CIs that had lower bounds of less than 1.0 (0.77 – 2.94 and 0.55 – 6.20). Eriksson et al (2008) noted that many glyphosate users had previously been exposed to MCPA, and suggested this as an explanation for why neither chemical showed a significant OR when subjected to multivariate analysis.

At best, the association between glyphosate and NHL in this study is equivocal, remains potentially confounded by established risk factors such as immunosuppression and Epstein-Barr virus (as noted previously by the Australian DoHA, 2005), and could also have been affected by recall, exposure measurement and information bias if NHL cases or their interviewers believed that their disease may be related to pesticides (Mink, unpublished). Mink has also observed that, by excluding 88 potential cases who died before they could be interviewed, the study

[24] In Hardell et al (2002), the next-of-kin provided information for deceased individuals.

population did not represent those cases with more aggressive disease. Furthermore, the dose-response analysis may have been confounded by exposure to other herbicides, and was based on unequal cut-off points for glyphosate (≤10 days or >10 days) and "other" herbicides (≤32 days or >32 days) (Mink, unpublished).

APPENDIX 5: PHARMACOKINETICS OF GLYPHOSATE AND ITS METABOLITE AMPA IN RATS

Anadon et al (2009): Laboratory grade glyphosate (Sigma Chemical Co, St Louis, MO, USA; purity 95%) was administered to male Wistar rats (Charles River Inc, Margate, Kent, UK; bw 200 – 210 g) at 100 mg/kg bw IV (in 0.1 mL glycerol formal) or 400 mg/kg PO (gavage to fasted animals in 0.5 mL corn oil). Groups of 8 rats were killed and exsanguinated at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h post-dosing, and the concentrations of glyphosate and aminomethyl phosphonic acid (AMPA) were measured in plasma by HPLC with fluorescence detection.

Glyphosate, IV administration: Following an initial peak concentration (C_{max}) of 166 µg/mL plasma pharmacokinetics were biphasic, consistent with a two-compartment open model, with rapid distribution and gradual elimination. The volume of distribution at steady state was 2.99 L/kg, suggesting extensive diffusion into the tissues. Clearance was 0.995 L/h/kg. The elimination half-life from plasma was 9.99 h and the area under the concentration vs time curve (AUC) was 100 mg.h/L.

Glyphosate, PO administration: Absorption from the GIT was gradual, with a C_{max} of 4.62 µg/mL occurring in plasma at 5.2 h. Oral bioavailability was poor (23.2%). Clearance was the same as following IV administration and the AUC was similar (at 93.3 mg.h/L), but the elimination half-life from plasma was appreciably more prolonged (14.4 h).

AMPA: The metabolite first appeared in plasma within 0.25 h of PO dosing, and had similar pharmacokinetic behaviour to glyphosate. The C_{max} (0.42 µg/mL) occurred at 2.4 h. An AUC of 6.1 mg.h/L was attained, *ca* 6.5% of glyphosate's AUC in plasma. The elimination half-life of 15.1 h was similar to that of the parent chemical after PO administration.