From: FARMER, DONNA R [AG/1000]
Sent: Thursday, November 18, 2010 1:50 PM
To: 'John DeSesso'
Subject: First half

John,

Attached is the first 46 pages.

I added a section in genotox from the Gasnier study...see a attached a critique we did that I took that from. Am working on a section for gasiner in the mechanistic section. Also we cut and pasted in summaries of the POEA surfactant studies. Attached are more detailed summaries – see Knapp. For right now I think we should go with POEA surfactants. I am checking to find out if there are any concerns with using MON 0818 and MON 8109 as well as indicating they are tallow and coco-derived – will get back to you on that as well as sending the remaining pages. Hope to have them done this afternoon if not will send tomorrow.
[EMBED Outlook.FileAttach][EMBED Outlook.FileAttach][EMBED Outlook.FileAttach]
Regards,

Donna

DRAFT

Developmental and Reproductive Outcomes in Humans and Animals after Glyphosate Exposure: A Critical Analysis ~~of the Available Literature~~

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Number of Tables: 12
Number of Figures: 0

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ABSTRACT

Glyphosate is the active ingredient of several widely used herbicide formulations including Roundup®, AquaMaster® and Vision® branded products. There is no one single "Roundup" product on the market today but rather a range of Roundup branded products such as Roundup WeatherMax, Roundup PowerMax and Roundup PROMAX. Glyphosate targets the shikimate metabolic pathway, which is found in plants but not in animals. Despite the relative safety of glyphosate, various adverse developmental and reproductive problems have been alleged as a result of exposure in humans and animals. To assess the developmental and reproductive safety of glyphosate, an analysis of the available literature was conducted. Epidemiological and animal reports, as well as studies on mechanisms of action related to possible developmental and reproductive effects of glyphosate, were reviewed. An evaluation of this database found no consistent effects of glyphosate exposure on reproductive health or the developing offspring. Furthermore, no plausible mechanisms of action for such effects were elucidated. Although toxicity was observed in studies that used glyphosate-based formulations, the data strongly suggest that such effects were due to surfactants present in the formulations and not the direct result of glyphosate exposure. To estimate potential human exposure concentrations to glyphosate as a result of working directly with the herbicide, available biomonitoring data were examined. These data demonstrate extremely low human exposures as a result of normal application practices. Furthermore, the estimated exposure concentrations in humans are many-fold less than the oral reference dose for glyphosate of 21.75/mg/kg/day set by the US Environmental Protection Agency (US EPA, 1993). Overall, the available literature shows no solid evidence linking glyphosate exposure to adverse developmental or reproductive effects at environmentally realistic exposure concentrations.

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INTRODUCTION

First approved for the broad spectrum control of weeds in 1974 (Franz et al, 1997), the applications for glyphosate have been expanded over the years, making it one of the most commonly used herbicides worldwide. Today, glyphosate-based herbicide formulations are used in over 100 countries, in almost all phases of agricultural, industrial, silvicultural, and residential weed control. In 2001, an estimated 85-90 million pounds of glyphosate were applied in the US agricultural sector alone, making it the number one pesticide active ingredient used in the US (Kiely et al., 2004). Glyphosate's rapid rise in popularity since its first introduction is not only because of its effectiveness in controlling the growth of invasive plants, but also in large part, due to its relative safety for humans and animals.

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Glyphosate acid is typically referred to as the technical grade material and because of its limited solubility in water, commercial formulations contain glyphosate in the form of a salt (i.e., potassium, isopropylamine, ammonium). In addition to glyphosate, commercial formulations typically include water and a surfactant system. Surfactant systems enable herbicide products to adhere to the surface of leaves allowing the penetration of the active ingredient (Franz et al., 1997)

In plants, glyphosate inhibits enolpyruvylshikimate phosphate synthase, an enzyme required for the synthesis of several essential aromatic amino acids (Franz et al, 1997). This metabolic pathway is common to all plants, making glyphosate an effective, non-selective herbicide. Because the shikimate pathway is not shared by members of the animal kingdom, glyphosate is not expected to adversely affect humans and other mammals under normal use conditions. While classified under the herbicide class phosphonomethyl amino acids, glyphosate is often mischaracterized as an organophosphate. This is likely due to the molecular structure being an organic molecule containing a phosphorus atom. However, clinical reports describing incidents of human ingestion of glyphosate do not reflect the classic symptoms for organophosphate poisoning; salivation, lacrimation, urination and defecation (SLUD). ~~In addition, g~~Glyphosate is not anticipated to persist in the environment for extended periods of time following application. ~~Glyphosate In addition g~~Glyphosate is nonvolatile and binds tightly to most soils, making it unlikely to migrate to groundwater or reach nontarget plants. Over time, it is degraded by microbes in soil and natural waters into substances such as carbon dioxide and phosphate (Giesy et al., 2000). These factors limit the degree of exposure of non-target species to glyphosate, thus further increasing its relative safety. Based on these facts, in addition to results from animal toxicity ~~exposure~~ studies, the US Environmental Protection Agency (US EPA), as well as other regulatory agencies worldwide, has deemed glyphosate to be of low to minimal toxicity to humans via reasonably anticipated exposure routes (US EPA, 1993).

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Potential routes for human exposure to glyphosate include inadvertent ocular exposure during herbicide mixing and application; dermal exposure due to mixing/application or contact with treated plants; and oral exposure through the ingestion of treated crops or contaminated water, although accidental ingestion of larger amounts by children and adults, and intentional ingestion for suicidal purposes have been reported. Inhalation of glyphosate is anticipated to be minimal because of the chemical's nonvolatility. In fact, the US EPA's registration requirement for an acute inhalation study was waived for glyphosate due its nonvolatile nature (US EPA, 1993).

~~Glyphosate absorption via all routes appears to be fairly limited.~~ Although data regarding the ocular absorption of glyphosate are not available, those related to dermal exposures indicate extremely low skin absorption rates. ~~In~~ In non-GLP studies conducted with rhesus monkeys, the measured absorption of glyphosate applied to the skin ranged from 0.4 – 2.2 % (Webster et al., 1991; Maibach, 1986), depending on the type of material applied to the skin (diluted or undiluted herbicidal formulation versus pure glyphosate), the duration of exposure, and the applied volume. *In vivo* and *in vitro* human dermal absorption studies have shown that <2% of Roundup is absorbed as either a concentrated or diluted spray (Webster et al., 1991; Franz, 1983). Several recent *in vitro* human dermal absorption studies have been conducted on glyphosate or glyphosate- based

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formulations. Bo Nielsen et al. (2009) tested a variety of compounds including glyphosate acid using an OECD 428 like design with 0.4% glyphosate absorbed through the skin and 0.7% recovered in or on the skin. Total glyphosate recovery was 92%. This study design was conservative in that (i) duration of exposure was a continuous 48 hours without an interim wash, rather than 24 hours duration with a 6-10 hour interim wash; (ii) no tape strips were taken off the skin to differentiate between potentially biologically available test item in the skin and the top *stratum corneum* layers which would be considered exfoliated over time; and (iii) test cells were occluded with parafilm (probably to prevent loss of other volatile test items in the study, but glyphosate is non-volatile), resulting in hydration of the skin and potentially enhanced dermal permeability. Ward (2010) conducted three OECD 428 studies on three different glyphosate--based formulations, testing concentrate and two dilutions representing the range of field concentrations for each of the three formulations, ranging from 12.5 X to 200X dilutions. These studies were conservative in that exposures were for a full 24 hours with no interim wash. Total glyphosate recovery in these experiments ranged from 98.6% to 106%. All experiments exhibited extremely low glyphosate biological availability (total absorbed + remaining in skin after tape stripping), ranging from less than 0.05% to 0.123% for the concentrates and less than 0.14% to 0.8% for the dilute formulations.

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Based on studies conducted in the rat, oral absorption also appears to be limited. Following administration of a single oral dose (5.6 mg/kg – 10 mg/kg), approximately 30% of the glyphosate is absorbed in the rat, as determined by measurements of the area under the curve for whole blood and urinary excretion data (Bradberry et al., 2004; Williams et al., 2000; Chan and Mahler, 1992). Oral absorption is further reduced to 19-23% by the application of extremely high doses of glyphosate (*e.g.*, 1000 mg/kg) and in repeat dosing regimens (Chan and Mahler, 1992; Williams et al., 2000). Glyphosate does not bioaccumulate to any appreciable levels (Brewster et al., 1991). Following ingestion of a relatively large dose in the rat, the plasma glyphosate concentrations peak at around four hours post-administration and are virtually undetectable by 12 hours (Bradberry et al., 2004). Furthermore, glyphosate is poorly metabolized in mammals and is quickly eliminated in the urine and feces unchanged (FAO, 2005).

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Despite the well established safety of glyphosate for humans by regulatory agencies, it has been suggested that chronic, low-level exposure may lead to developmental and reproductive health problems (~~Dallegrave et al., 2003, 2007; Marc et al., 2005; Benachour et al., 2007~~), particularly for men and women residing in agricultural areas associated with heavy herbicide use. This notion has developed based primarily on results from recently published *in vitro* and animal studies conducted using glyphosate-based herbicide formulations(Dariuch et al., 2001; Dallegrave et al., 2003, 2007; Marc et al., 2005; Richards et al., 2005; Benachour et al., 2007, Benachour et al., 2009; Gasnier et al., 2009; Romano et al., 2010; Pagenelli et al., 2010,. In order to determine whether appropriate data exist to support this claim, a thorough evaluation of the scientific literature was conducted. Experimental investigations conducted by the Monsanto Company in

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support of regulatory requirements were made available to the authors. These studies were compliant both with contemporary regulatory guidelines and Good Laboratory Practices (GLPs). In addition, research reports published in the open scientific literature were identified through automated searches of PubMed, SciFinder, and ToxLine. Critiques of all the reviewed studies are included herein, along with rationales for using or discounting the reported results for the purpose of human health risk assessment. Finally, assessments published by international organizations and regulatory agencies were examined as supporting documentation. Emphasis was placed on identifying potentially adverse reproductive health and developmental effects; however, a review of available biomonitoring data was also conducted to understand anticipated exposure levels for humans.

ASSESSMENT OF POTENTIAL REPRODUCTIVE HEALTH AND DEVELOPMENTAL EFFECTS

Humans are the primary focus for this evaluation of possible reproductive and developmental effects. Consequently, published epidemiology studies addressing the potential for such outcomes upon exposure to glyphosate-containing herbicides were first assessed. Next, animal studies (both published reports as well as unpublished studies ~~conducted~~ owned by Monsanto) addressing appropriate toxicity endpoints were reviewed. Finally, published mechanistic studies using glyphosate and glyphosate-based herbicidal formulations were evaluated to determine whether a plausible mechanism of action could be established to explain how glyphosate could contribute to reproductive and/or developmental problems in humans and other mammals.

Epidemiological Evaluation

Although a substantial body of data exists regarding the adverse reproductive and developmental effects of pesticide exposures in general, very few epidemiology studies have been conducted to specifically assess the potentially adverse effects associated with glyphosate exposure. Based on a review of the scientific literature, only ten epidemiology studies evaluating pregnancy outcomes or reproductive health as they relate to glyphosate exposures were identified (Tables 1 and 2). None of these was designed to specifically assess exposures to glyphosate; rather, these studies address pesticide and/or herbicide exposures, categorized by use or chemical group. In these publications Gglyphosate is only mentioned in passing, or grouped with other herbicides such as phosphonamino herbicides ~~(of which glyphosate-containing herbicides are only a small portion)~~ or organophosphate pesticides (an incorrect classification of glyphosate).

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Developmental Effects

The Ontario Farm Family Health Study (OFFHS) was initiated in 1990/1991 to retrospectively examine the possible associations between various pesticide exposures and adverse developmental and reproductive outcomes. Based on the 1986 Canadian Census of Agriculture, 7,379 farms in Ontario were identified as likely

to be full-time family-run operations. Through telephone screening a subset of 2,946 couples was identified as eligible for study based on residence on or near the farm year-round and the female partner being ≤ 44 years of age. A total of 1,898 couples provided completed questionnaires from the farm operator, husband, and wife (response rate of 64%). These couples reported a total of 5,853 pregnancies. Pregnancy outcomes were determined through maternal self reports. Based on the OFFHS, three separate papers investigating the adverse effects of glyphosate exposure on prenatal development or reproductive health have been published (Savitz et al., 1997; Curtis et al., 1999; Arbuckle et al., 2001). Other papers published from the OFFHS either did not address potential effects of glyphosate exposure or did not examine prenatal development and/or reproductive health.

Pregnancy Outcome

In Savitz et al. (1997), data from the OFFHS were used to examine the possible association of male pesticide exposure with adverse pregnancy outcomes. Out of a total of 5,853 OFFHS pregnancies, 3,984 were included in this study. Approximately 40% of those included in the analysis occurred ten years or more before the study commenced, which likely introduces some recall bias. The majority of those pregnancies not included in the study were omitted because the pregnancy did not occur while in residence on the farm. Pregnancies were classified according to outcome (single live birth, miscarriage, stillbirth, preterm, small for gestational age [SGA], etc.), but were not confirmed through medical records. Males were asked about their farm activities over the past five years. Activities involving the mixing and/or application of crop herbicides, crop insecticides or fungicides, livestock chemicals, yard herbicides, and/or building pesticides met the study requirements for direct pesticide exposure. All reported activities were assumed to extend backwards beyond the five-year period covered by the questionnaire. Each pregnancy was classified as exposed or not exposed based on whether the male partner partook in an activity involving pesticide exposure for one or more months during the three months prior to the time of conception or during the month of conception itself. Based on information provided by the farm operators, the analyses were further refined, taking into consideration the specific pesticides used on the farms during the preconception/conception period. These methods for exposure assessment likely introduced substantial exposure misclassification, especially for those pregnancies that occurred prior to the five-year period covered by the questionnaire. Substantial recall bias is also likely since fathers of pregnancies with adverse outcomes are more likely to recall pesticide use during the preconception period than fathers of pregnancies with normal outcomes. Study authors controlled for potential confounders (including age of parents, level of education, jobs outside the farm, smoking habits, alcohol consumption, caffeine use, etc.) in their analyses, as appropriate. The study results show that glyphosate exposure of males was not associated with an increased risk of miscarriage, preterm delivery, or SGA delivery by their female partners.

Spontaneous Abortions

Using the OFFHS data, Arbuckle et al. (2001) further dissected out the influence of pre- and post-conception pesticide exposures on spontaneous abortion, or miscarriage, risks. Self-reported miscarriages of less than 20 weeks of gestation were divided into two groups: those of less than 12 weeks of gestation and those occurring between weeks 12 and 19 of gestation. Out of a total of 3,936 pregnancies, 395 spontaneous abortions were reported; all but five were reported to have been medically confirmed and 57% occurred before 12 weeks of gestation. The approximate 10% rate for spontaneous abortions reported in this study is substantially lower than the ~12-25% rate reported for the general population (Everett, 1997; Wilcox et al., 1999). Recall was greater than five years for 64% of the spontaneous abortions and greater than ten years for 34% (Arbuckle et al., 1999), thus, allowing for some recall bias, as previously discussed. For each pregnancy, a monthly agricultural and residential pesticide use history was constructed from questionnaire data provided by the farm operator, husband, and wife. Pesticide exposures were analyzed using two exposure windows: the preconception period (defined as the three calendar months prior to conception and the month of conception, combined) and the post-conception period (defined as the three calendar months of the first trimester). Although not specifically discussed in this paper, it is likely that the exposure data were extrapolated in part, as was done in Savitz et al. (1997) and Curtis et al. (1999; discussed later); thus, substantial exposure misclassification may have occurred. Because strong confounding variables were not apparent from previous analyses of the data (Arbuckle et al., 1999), only crude odds ratios (ORs) were calculated. Preconception exposure for fathers to glyphosate showed an elevated risk for spontaneous abortions between weeks 12 and 19 of gestation, although this risk was not statistically significant (OR = 1.7, 95% confidence interval [CI] = 1.0–2.6). No other pre- or post-conception exposures to glyphosate demonstrated an increased risk of spontaneous abortions either before 12 weeks or between weeks 12 and 19 of gestation. A classification and regression tree analysis was used to explore possible statistical interactions between exposures and various risk factors for spontaneous abortion. No statistically significant interactions for glyphosate exposure were apparent. Overall, ~~although~~ the results ~~are suggestive, they~~ fail to demonstrate a significant association of glyphosate exposure with the risk of spontaneous abortion and should be considered cautiously in light of the substantial exposure misclassification that is likely to have taken place and the lack of adjustment made to the ORs to account for the influence of confounding variables.

Fetal Deaths

Bell et al. conducted two case-control studies to examine the possible associations between maternal pesticide exposure and fetal death due to congenital anomalies (Bell et al., 2001a) or other causes (Bell et al., 2001b). In the congenital anomalies case-control study (Bell et al., 2001a), 73 cases were identified from the 1984 vital statistics records of ten California counties. Cases were limited to fetal deaths identified as due to congenital anomalies and occurring after 20 weeks of gestation; these included 43 neonatal deaths within the first 24 hours after birth. All 611 controls were randomly selected from normal live births occurring in 1984 and frequency matched with cases by county and maternal age. Sites of pesticide application in the ten-county

area for 1983-1984 were determined from the California Pesticide Use Report database and linked to maternal addresses according to their township, range, and section (TRS – a unique location identifier used by the Public Land Survey System and equal to one square mile in area). In the narrow definition of exposure, a pregnancy was considered to be exposed if pesticides were applied to land within the same TRS as the maternal address. In the broader definition, a pregnancy was considered to be exposed if pesticides were applied within any of the eight surrounding TRSs or the same TRS as the maternal address. Exposures were assigned for each day of each woman's pregnancy for 327 different pesticides, which were categorized into five separate classes (phosphates, carbamates, pyrethroids, halogenated hydrocarbons, and endocrine disruptors). Glyphosate (referred to as "glyphosphate" in the paper) was classified as a phosphate/thiophosphate/phosphonate pesticide (by far, the broadest pesticide category evaluated in the study). Exposures were then analyzed according to three different periods of gestational age: exposure during gestational weeks 1-20; exposure during gestational weeks 1-13; and exposure during gestational weeks 3-8. A fourth exposure definition was also implemented, which further restricted the definition of non-exposure to be no exposure to any of the five classes of pesticides during gestational weeks 3-8. Although this method of assigning exposure is not subject to recall bias (a major strength), its still allows for some misclassification. For example, if the home was upwind of the pesticide being applied, then it likely would not have been exposed, despite being within the same or an adjoining TRS to the maternal address. Also, pregnancies that were exposed only once during a particular exposure period would be lumped with those that were exposed multiple times during the same time period.

Using the broad definition of exposure (*i.e.*, pesticide exposure in the maternal TRS or one of the eight adjoining TRSs), no statistically increased odds of fetal death were associated with exposure to phosphates during any of the exposure periods examined. In contrast, a statistically significant risk of fetal death due to congenital anomalies was associated with exposure to pyrethoids (discussed in erratum Bell et al., 2001c) and halogenated hydrocarbons. These risks increased as the definition of the exposure period was tapered down from anytime during weeks 1-20 to only during weeks 3-8 of gestation. Using the narrow definition of exposure (within the same TRS as the maternal address), findings for halogenated hydrocarbons and pyrethoids lost statistical significance. Exposure to phosphates, on the other hand, showed a statistically-significant association with fetal death due to congenital anomalies, which increased as the definition of exposure became more limited. That is, phosphates exposure during weeks 1-20 of gestation was associated with an OR of 2.0 (95% CI = 1.0 – 4.0), while exposure during weeks 3-8 only was associated with an OR of 3.0 (95% CI = 1.4 – 6.5). It is not clear what conclusions, if any, can be drawn from these study results with regards to glyphosate exposure. Results specific to glyphosate are not available, and as previously mentioned, glyphosate is only one of the many pesticides included in the broad phosphates category. In fact, the vast majority of pesticides included in this category are organophosphate insecticides, which act by phosphorylating the acetylcholinesterase enzyme of insects and mammals – a mechanism of action entirely different from that of

glyphosate, which targets an enzyme found only in plants and some microorganisms. This suggests that glyphosate was inappropriately included in the phosphates category, and further, that a risk for congenital anomalies cannot be inferred for glyphosate exposure.

In the case-control study of fetal death due to other causes (Bell et al., 2001b), 314 cases were identified from the 1984 vital statistics records of the same ten California counties as the previous study. These cases included 86 neonatal deaths within 24 hours of birth, but excluded deaths for pregnancies of less than 20 weeks, those due to congenital abnormalities, and other causes not likely to be related to environmental exposures (i.e., multiple births, umbilical cord compression, etc.). Controls were identified from normal live births in 1984 and frequency matched by maternal age and county. As in the previous study (Bell et al., 2001a), there were 611 controls; however, it is not clear if these controls are the same in both studies. Exposures were determined according to the same methods used in the previous study (and thus, are subject to the same misclassification issues), and analyzed according to trimester and month of gestation. Because fetal deaths that occurred at a later gestational age had greater opportunity to be exposed than those that occurred earlier on, analyses were adjusted for gestational length. Adjusted hazard ratios and 95% CIs were calculated. Overall, no pesticide class was statistically associated with fetal death using either the broad or narrow definitions of exposure as analyzed according to trimester.

Birth Defects

Garry et al. (2002) conducted a cross-sectional study to examine the reproductive health outcomes of pesticide applicators and their families. Approximately 3,000 residents of the Red River Valley of Minnesota were licensed to apply pesticides from 1991 to 1996. Half were randomly selected for study, and of these, 1,070 volunteered to participate. Enough detailed information regarding reproductive health outcomes and pesticide use was obtained for 695 families (536 with children) and 1,532 live births. Births occurred from 1968 to 1998, with over half of the births occurring before 1978 (almost 20 years prior to the study's initiation). Parent-reported health information (obtained through written questionnaire) was confirmed through birth certificates and medical records, when possible. Information regarding pesticide use was obtained initially via telephone survey, and approximately six months later, by written questionnaire. Seventy children with congenital birth defects were identified. Parents of children with birth defects did not differ from parents of children without birth defects in terms of age at time of child's birth, smoking status, or consumption of alcoholic beverages. Interestingly, the frequency of birth defects identified during the first year of life as reported in this study was much higher than that observed in an earlier cohort study that only used medically-confirmed cases of birth defects (Garry et al., 1996). This result suggests that the method for study subject selection was biased in the cross-sectional study in such a way as to over-represent families of children with birth defects. Pesticide exposure was assessed according to specific classes of use (herbicide only; herbicide and insecticide; herbicide, insecticide and fungicide; herbicide, insecticide and fumigant; and use of all four pesticide classes), with use of herbicides-only being the referent group for comparison purposes. Use of all four classes of

compounds was associated with a slightly increased incidence of having children with birth defects compared to use of herbicide alone; however, the association did not reach statistical significance. Although the study authors clearly indicated that developmental neurobehavioral disorders would not be considered in their detailed analyses due to the lack of uniformity in such diagnoses, a detailed analysis was preformed. The study authors reported that 43% (6/14) of children with parent-reported attention deficit disorder (ADD)/attention deficit hyperactivity disorder (ADHD) had parents that used phosphonamino herbicides, with an OR of 3.6 (95% CI = 1.35 – 9.65). Glyphosate and "Roundup" [specific formulation not specified] were the only herbicides in this class mentioned by name. No other data related to glyphosate and "Roundup" were reported. No conclusions can be drawn from this finding. Only 14 cases of ADD/ADHD were reported in a total of 1,532 live births – an incidence substantially lower than the 7% reported for the general population (Bloom and Dey, 2006). Additionally, these are parent-reported cases that have not been confirmed through medical records and, as the authors themselves stated, such diagnoses can be highly unreliable. Without proper ascertainment of these neurobehavioral disorders, and in light of the fact that their incidence was lower than that normally observed in the general population, this finding provides no insight concerning the potential developmental health effects of glyphosate. Other papers published based on the Red River Valley cohort either did not address potential effects of glyphosate exposure or did not examine reproductive health and/or developmental issues.

Neural Tube Defects

Rull et al. (2006) assessed pesticide exposure using two population-based case-control studies of neural tube defects (NTDs) conducted by the California Birth Defects Monitoring Program (Shaw et al., 1995 and 1999). Cases were confirmed diagnoses of anencephaly, spina bifida cystica, craniorhachischisis, and iniencephaly in Californian infants and fetuses delivered between 1987 and 1991. Controls included a random sample of normal singleton births and fetal deaths from the same time frame. Residential, medical, reproductive, occupational, nutritional and family histories were taken by telephone (on average of 3.8 years after date of delivery; Shaw et al., 1999) or in-person interviews (on average 5 months after delivery date; Shaw et al., 1995). Maternal addresses during the calendar month of conception and the month following conception were geo-coded to latitude and longitude coordinates, and buffer zones of 500- and 1,000-meter radii were determined as potential exposure zones. This information was then used to determine possible pesticide exposures based on pesticide-use report data from the California Department of Pesticide Regulation. The risk of NTDs was estimated for 59 specific pesticides using both single- and multiple-pesticide exposure models, taking into consideration the potentially confounding variables of ethnicity, education level, smoking status, and vitamin use. A hierarchical logistic regression model was also run to minimize the number of false-positive results due to simultaneous analysis for multiple pesticides. Risks for anencephaly and spina bifida, specifically, were also calculated according to pesticide class. As in Bell et al. (2001a, b), glyphosate was inappropriately categorized as an organophosphate pesticide; however, NTD risks were calculated for each

pesticide separately rather than by pesticide class. NTD risks were increased for foreign-born Latina mothers, those who did not complete high school, and mothers who did not take vitamins. Glyphosate exposure during the peri-conception period was not associated with an increased risk of NTDs (single pesticide model: OR = 1.5, 95% CI = 1.0 – 2.4; multiple-pesticide model: OR = 1.5, 95% CI = 0.8 – 2.9; hierarchical logistic regression model: OR = 1.4, 95% CI = 0.8 – 2.5).

Reproductive Health

Fertility

Curtis et al. (1999) conducted a retrospective cohort study to look at possible effects of pesticide exposure on fecundability. The study design was the same as that of Savitz et al. (1997), and based on the same 1,898 couples and 5,853 pregnancies identified in the OFFHS. Only planned pregnancies, for which women noted discontinuing a method of birth control in order to conceive, were evaluated. These included 2,012 pregnancies, 67% of which were conceived at least five years prior to the study and 36% of which occurred at least 10 years prior. The substantial amount of time that exists between the time of conception and the start of the study for the majority of these pregnancies may have introduced some recall bias, although the study authors indicated that an analysis limited to those pregnancies conceived within five years of the study gave similar results as those obtained through an analysis based on all of the pregnancies combined. Detailed reproductive histories were not taken to confirm time to pregnancy claims or to explore other factors that could influence fecundability (e.g., frequency of intercourse, breastfeeding history, reproductive health issues, menstrual cycle characteristics, etc.). Through completed questionnaires provided by farm operators, husbands and wives, monthly histories of pesticide usage were constructed for each farm and extrapolated back to years prior to 1991 (the year to which the questionnaire specifically referred). Because it assumes that practices do not change on the farm over time and that all pesticide-related activities occur at the same time each year, this method of determining exposures lends itself to substantial exposure misclassification. For each pregnancy, exposure to certain classes of pesticides, as well as specific pesticide chemicals (including glyphosate), was determined on a yes/no basis for each month that the couple tried to conceive and the two months prior. Exposures were also classified by whether the husband, wife, or both participated in a pesticide activity. For each exposure type, the authors calculated a conditional fecundability ratio (CFR) – that is, the ratio of probabilities of conceiving in any given month for the exposed group over that of the unexposed group. Results showed that 75% of the planned pregnancies were conceived in three months or less. Reduced fecundability was associated with women's exposure to glyphosate (regardless of men's activity), but this association was not statistically significant (CFR = 0.61, 95% CI = 0.30 – 1.26). Also, the finding may be related to factors other than pesticide exposure, such as the influence of heavy manual labor on the menstrual cycle, or reduced frequency of intercourse due to a heavier than normal workload. In contrast to the above finding, glyphosate use on the farm (with no reported pesticide-related activities by women or men) was

associated with an increased, albeit not statistically significant, fecundability (CFR = 1.26, 95% CI = 0.94 – 1.69). Also, men's exposure to glyphosate demonstrated a statistically significant increase in fecundability (CFR = 1.30, 95% CI = 1.07 – 1.56); however, this association was downplayed by the authors as a random chance finding. Overall, Curtis et al. (1999) found no significant adverse effects on time to pregnancy associated with glyphosate exposure. Regardless, the study is severely limited by the likelihood of widespread exposure misclassification and the exclusion of information related to common factors that affect fecundability.

Larsen et al. (1998) investigated whether time to pregnancy was associated with the use of pesticides during farming practices. A total of 904 male farmers ranging in age from 18-50 years and living in the Jutland were identified from the Danish Ministry of Agriculture lists of traditional and organic farmers. Information on the cohort was collected via telephone interviews between October 1995 and May 1996. Reproductive histories focused primarily on questions related to the youngest child. Information was also collected on potential confounders such as last contraceptive method, smoking habits, age, and female parity. Participants were divided into four exposure groups according to their pesticide use in the year before the youngest child was born; traditional farmers spraying pesticides (n = 450), traditional farmers who did not spray the pesticides themselves (n = 72), organic farmers (n = 94), and those not involved in farming at the time the youngest child was conceived (n = 66); this last group was excluded from the final analyses. Those farmers never married (n = 36), without children (n = 97), or whose youngest child was conceived due to failure of the birth control method (n = 89) were also excluded from the final analyses. Farmers actively involved in spraying pesticides were asked about the number of hectares treated, type of tractor and spraying equipment, use of protective equipment, and type of crops. These farmers were then assigned to three index groups based on this information. Cumulative potential exposures were determined based on the total number of years of pesticide use. From a list of possible pesticides, farmers were asked to identify those pesticides used in the year prior to the youngest child's birth. Glyphosate was considered a potentially spermatotoxic pesticide by the researchers, although the basis for this assumption was not provided. Time to pregnancy data for the organic farmers and two groups of traditional farmers were analyzed using a Cox regression model. For reasons that are not clear in the paper, those showing time to pregnancy greater than 12 months were excluded from the evaluations. The fecundability ratio (FR) between traditional farmers who actively participated in pesticide application and organic farmers was 1.03 (95 % CI = 0.75 – 1.40). Interestingly, increased cumulative exposure to pesticides was associated with a significantly decreased time to pregnancy, but only in the 11-15 years category (FR = 1.61, 95 % CI = 1.07 – 2.40). Also, the use of three or more "spermatotoxic" pesticides in the year prior to the youngest child's birth had no significant effect on the time to pregnancy (FR = 0.88, 95 % CI = 0.66 – 1.18). No information specific to glyphosate was presented. Because recall was greater than five years for 52 % of the traditional farmers who sprayed pesticides, these data likely suffer from some exposure misclassifications. Additionally, because those without children and those with greater than 12 months to pregnancy were excluded from the analyses, it is possible that an effect of pesticide exposure could have been underestimated.

Greenlee et al. (2003) conducted a case-control study to determine risk factors for female infertility in an agricultural region. Cases were women, ages 18-35 years, who sought treatment at an OB/GYN department in Wisconsin between June 1997 and February 2001 for either primary or secondary infertility. (Infertility was defined as experiencing at least 12 months of unprotected intercourse without conceiving a pregnancy ending in a live birth). Cases of infertility due to surgical causes or male infertility were excluded from the study. Controls were women, ages 18-35 years, seeking prenatal care in their first trimester of pregnancy at the same OB/GYN department during the same period of enrollment and who conceived in less than 12 months of trying; those reporting ever having trouble conceiving or maintaining a pregnancy were excluded. Controls were frequency-matched with cases based on age and clinic service date for a total of 322 cases and 322 controls. Data were gathered by telephone interview regarding activities during the two years prior to the reported pregnancy attempt dates. Before the interview, subjects were provided with a list of pesticides and possible exposure scenarios to review. Based on logistic regression models, cases and controls were similar in age; however, infertile women tended to work outside the home more, be less educated, to smoke and consume more alcoholic beverages, to have gained weight steadily during adulthood, and to have older male partners than controls. Cases also spent significantly more time reviewing the pesticide exposures list than did controls (29.3 versus 18.5 minutes, respectively), indicating likely recall bias. An analysis of agricultural variables showed that a woman's exposure to herbicides at any time during the two-year period prior to trying to conceive was statistically associated with an increased risk of infertility. Interestingly, this variable only reached statistical significance after adjusting for confounding variables (crude OR = 2.3, 95% CI = 0.9 – 6.1; adjusted OR = 26.9, 95% CI = 1.9 – 385). The large change in the odds ratio following adjustment, especially in light of the lack of substantial effect on the other agricultural variables evaluated, seems suspect. Furthermore, it was based on small numbers of cases (21/322) and controls (13/322), indicating that the vast majority of both had no exposure to herbicides during the period of concern. While glyphosate was reported to be the most commonly used herbicide by both cases and controls (54 and 36 women, respectively), it is unclear why these numbers are greater than numbers of cases and controls reporting use of herbicides during the two-year period of interest. Based on questionable data and the fact that glyphosate use, specifically, was not evaluated, no conclusions can be drawn from this study regarding glyphosate's association with female infertility or lack thereof.

Comment [D9]: I would say possible recall bias. Three lines about you note this group was less educated, which may also account for the increased time to complete reading & responding.

Menstrual Cycle Characteristics

The Agricultural Health Study was a prospective cohort study conducted on over 50,000 pesticide applicators and more than 32,000 of their spouses from North Carolina and Iowa. Data on the cohort were collected either by written questionnaire or by telephone between 1993 and 1997. Based on these data, Farr et al. (2004) examined the effects of pesticide use on the menstrual cycle characteristics of 3,103 women. The cohort was limited to female private pesticide applicators or the spouses of private pesticide applicators who completed

the Female and Family Health questionnaire, were identified as pre-menopausal and between the ages of 21 and 40 years, had a body mass index between 15 and 40, and were not pregnant, breastfeeding, or taking oral contraceptives. Five menstrual cycle characteristics were assessed: short cycles (24 days or less), long cycles (36 days or more), irregular cycles, missed periods (no periods for an interval of more than six weeks in the last 12 months), and bleeding/spotting between periods within the last 12 months. Women were asked about their use of 50 different pesticides, including the average number of days per year they mixed and applied these pesticides. Women were grouped into three exposure categories based upon their responses: 0 days, 1-9 days, and 10 or more days. For analysis purposes, the pesticides of interest were grouped into three categories: endocrine disruptors, those with ovarian effects, and estrous cycle disruptors. Glyphosate was listed as a pesticide with ovarian effects, although the basis for this classification is not clear. Women who reported ever mixing and applying pesticides were less likely to have irregular periods (OR = 0.55, 95% CI = 0.41 – 0.75), but more likely to report missed periods (OR = 1.6, 95% CI = 1.3 – 2.0). Controlling for the average number of days worked in the fields slightly buffered these findings (ORs changed from 0.55 and 1.6 to 0.61 and 1.3, respectively). Limiting the analyses to those women exposed to probable/possible hormonally active or ovotoxic pesticides only slightly strengthened the associations. Findings for some specific pesticides were reported; however, no significant associations were noted for glyphosate exposure. Other papers published from the Agricultural Health Study either did not address potential effects of glyphosate exposure or did not examine reproductive health/development issues.

Summary – Epidemiology Studies

As previously mentioned, the body of epidemiological data regarding potentially adverse reproductive health or pregnancy outcomes associated with glyphosate use is scant. Only ten such studies were identified, and none of these was designed to assess the effects of glyphosate exposure, specifically. Furthermore, all of these studies suffer from likely exposure misclassifications, as previously discussed. Of the six studies examining potential developmental effects, only Garry et al. (2002) claimed to observe a possible adverse outcome (increased risk of ADD/ADHD) associated with phosphonamino herbicide exposure of the parents of affected children. However, the diagnostic criteria for these neurobehavioral disorders are notoriously inconsistent, none of the study cases was confirmed through a review of medical records, and the study's ~~self~~parent-reported incidence of ADD/ADHD is lower than that of the general population. Thus, this study should be considered uninformative. In Bell et al. (2001a), parental exposure to phosphate pesticides was statistically associated with an increased risk of fetal death due to congenital anomalies; however, the authors had inappropriately included glyphosate in this class of chemicals. Therefore, no conclusions regarding glyphosate exposure can be drawn from this analysis. None of the other developmental outcomes studies reported a statistically increased risk of adverse pregnancy outcomes associated with glyphosate exposure (Savitz et al., 1997; Arbuckle et al., 2001; Bell et al., 2001b; and Rull et al., 2006). Of four studies that assessed the potential

reproductive health effects of glyphosate (Larsen et al., 1998; Curtis et al., 1999; Greenlee et al., 2003; and Farr et al, 2004), none reported adverse outcomes associated with exposure. Greenlee et al. (2003) suggested that female infertility may be influenced by herbicide exposure, but the data are questionable and too few exposed women were included in the study to address glyphosate exposure. In conclusion, although the database addressing these health issues is extremely limited, the totality of availability of epidemiological evidence fails to link glyphosate exposure with adverse reproductive health or pregnancy outcomes.

Animal Studies

Twelve animal studies assessing the potential developmental or reproductive health outcomes associated with glyphosate exposure were identified. Some of these studies tested pure glyphosate, while others tested commercial herbicide formulations, the formulation surfactant alone, or the major environmental breakdown product of glyphosate (aminomethylphosphonic acid: AMPA). These studies also varied greatly in their quality. Some studies were conducted under good laboratory practices (GLP compliant) and/or according to the health effects testing guidelines set by the US EPA or OECD; others used limited numbers of animals per group, inadequate study designs, and inappropriate controls and test materials were inadequately identified. For the purposes of review, these studies are grouped according to the specific test agent used.

Developmental Studies

Glyphosate

Two developmental toxicity studies were conducted using ~~pure~~ glyphosate ~~technical acid~~ (Table 3). Although these studies were conducted prior to the establishment of GLP, they both received quality assurance audits by the testing facility and were essentially guideline-compliant. In the first study by the International Research and Development Corporation (IRDC,1980a), pregnant female Charles River COBS®CD® rats were dosed once daily by gavage with 0, 300, 1,000, or 3,500 mg/kg/day glyphosate on gestational days (GD) 6-19 (25 animals per group). Individual doses were determined based on GD 6 body weights. Animals were examined daily for clinical signs of toxicity and body weights were recorded at appropriate intervals. Food consumption was not reported. On GD 20, all surviving animals were sacrificed and the developmental effects of treatment were assessed. The number of corpora lutea, implantations, resorptions, live and dead fetuses were recorded. Dams were examined for gross morphological changes of internal organs. Fetuses were weighed, sexed, and examined for malformations and variations. Approximately half of the fetuses were fixed in Bouin's solution for visceral examination via sectioning; the other half was prepared for evaluation of skeletal morphology. Gross malformations were not reported separately from soft tissue and skeletal malformations.

Comment [drf10]: Probably need to be consistent ...glyphosate or glyphosate acid or glyphosate technical grade ...or maybe best to check reports/publication and use what the authors used.

Comment [D11]: Tables 3-8 not presented

Clinical signs including soft stools, diarrhea, breathing rattles and inactivity were noted in rats dosed with 3,500 mg/kg/day of glyphosate. Six of 25 rats in this group died before the end of study. Red matter around the nose, mouth, forelimbs, and dorsal head were noted in animals prior to death, and stomach hemorrhages were noted in two of the six rats at necropsy. Statistically significant decreases in the mean numbers of implantations and viable fetuses per dam in the 300 mg/kg/day treatment group were noted; however, because no effects of treatment were noted in the next higher dose group of 1,000 mg/kg/day (and implantations occurred before treatment), these changes were attributed to random chance. In the 3,500 mg/kg/day glyphosate treatment group, a statistically significant increase in the number of resorptions, significant decreases in the mean numbers of implantations and viable fetuses per dam, and diminished mean fetal body weights were observed compared to controls. A slight, but not statistically significant, decrease in the mean number of corpora lutea per dam was also noted in this treatment group. Because ovulation and implantation occurred prior to dosing, the differences in corpora lutea and implantations were not considered to be related to glyphosate treatment.

No malformations were observed in fetuses from the 300 and 1,000 mg/kg/day treatment groups. Two control fetuses had soft tissue malformations and one had a skeletal malformation; these were found in a total of three different litters. In the 3,500 mg/kg/day glyphosate group, ten fetuses had malformations; these included seven soft tissue malformations and nine skeletal malformations. Upon closer examination, however, it is noted that these malformations were primarily minor, and included dwarfish and bent tails. Further, the malformations occurred in only three litters, with the same anomaly often presenting itself multiple times in a single litter (data not shown), suggesting a litter effect of genetic origin. An increased incidence of unossified sternebrae (a variation) was also reported in fetuses at 3,500 mg/kg/day. Overall, this study indicates a no observable adverse effect level (NOAEL) of 1,000 mg/kg/day glyphosate for both maternal and developmental toxicity.

In IRDC (1980b), female Dutch Belted rabbits were inseminated on GD 0 using semen from four proven male rabbits. Impregnated does were administered 0, 75, 175, or 350 mg/kg/day glyphosate by gavage on GD 6-27 (16 rabbits per group). Animals were examined daily for mortality and clinical signs of toxicity. Body weights were recorded at appropriate intervals. Food consumption rates were not recorded. Dams that did not survive to the end of study were necropsied to determine cause of death. All surviving animals were sacrificed on GD 28. The numbers of corpora lutea, implantations, resorptions, live and dead fetuses were recorded. All fetuses were weighed, sexed internally, examined for external and visceral malformations (via dissection), and prepared for skeletal examination using alizarin red. Gross malformations were not reported separately from soft tissue and skeletal malformations in this study. Soft stools and diarrhea were noted in all treatment groups, but showed a dose-dependent increased incidence in dams treated with 175 and 350 mg/kg/day glyphosate compared to controls. Animals at 350 mg/kg/day also demonstrated an increase in nasal discharge. Maternal body weight changes were highly variable across groups throughout the study and no significant differences compared to controls were noted. Abortions occurred in two rabbits from the control group, and in one rabbit in

each of the 175 and 350 mg/kg/day treatment groups. The reason for the relatively high abortion rate among control animals (2/16) is not known and was not discussed in the original study report. One, two, and 10 rabbits died before the end of study in the 75, 175, and 350 mg/kg/day glyphosate treatment groups, respectively. The causes of maternal death were determined for five of the 13 animals, but were not consistent across the group. A statistically significant increase in the number of viable fetuses per dam treated with 75 mg/kg/day was noted, but this result was considered to be a random occurrence because it was not observed in the two higher treatment groups. Compared to controls, glyphosate treatment had no effect on the numbers of corpora lutea, implantations, resorptions, live and dead fetuses, fetal sex ratios, or fetal weights. Glyphosate treatment also had no effect on the incidence of fetal malformations and variations. Based on these results, 175 mg/kg/day is considered the NOAEL for maternal toxicity, based on mortality and clinical signs at 350 mg/kg/day. Although no developmental toxicity was observed at any dose, 175 mg/kg/day is considered the NOAEL for developmental toxicity as well because too few fetuses were available at the high dose of 350 mg/kg/day for adequate toxicological assessment.

POEA (polyoxyethylenealkyl~~ethoxylated tallow amines~~) - a class of surfactants used in a number of commercial herbicide formulations

One GLP- and guideline-compliant developmental toxicity study has been conducted ~~using~~ with ~~a tallow derived~~ POEA (Holson, 1990; Table 3). Pregnant female Sprague Dawley Clr:CD©BR rats were dosed once daily by gavage with 0, 15, 100, or 300 mg/kg/day POEA on GD 6-15 (25 animals per group). Animals were examined daily for clinical signs of toxicity, and body weights and food consumption were recorded at appropriate intervals. On GD 20, animals were sacrificed and any developmental effects of treatment on the resulting offspring were determined by fresh visceral dissection and alizarin-staining for osseous effects. In this study, 6/25 animals treated with 300 mg/kg/day POEA died before the end of study and clinical signs of maternal toxicity were observed in the remaining animals at this dose level. Additionally, significant decreases in weight gain and food consumption during the treatment period were observed. Infrequent signs of clinical toxicity and significantly decreased food consumption on GD 6-9 were recorded for animals in the 100 mg/kg/day treatment group; however, despite these obvious signs of maternal toxicity, no developmental effects of treatment on the offspring were observed. From these results, the authors concluded that 15 mg/kg/day POEA was the NOAEL for maternal toxicity and 300 mg/kg/day POEA was the NOAEL for developmental toxicity.

AMPA (aminomethylphosphonic acid) – Major environmental breakdown product of glyphosate

One GLP- and guideline-compliant developmental toxicity study tested AMPA (Holson, 1991; Table 3). Pregnant female Sprague Dawley Crl:CD©BR rats were dosed once daily by gavage with 0, 150, 400, and 1,000 mg/kg/day AMPA on GD 6-15 (25 animals per group). Animals were examined once daily for clinical signs of toxicity, and body weights and food consumption recorded at appropriate intervals. On GD 20, animals

were sacrificed and the effects of treatment on development of the resulting offspring were recorded. Increased incidences of mucoid feces, soft stools, and hair loss were observed in animals treated with 400 and 1,000 mg/kg/day AMPA and these effects appeared to increase in a treatment-related manner. Offspring were examined by fresh visceral dissection and alizarin-staining for osseous effects. No developmental effects of treatment on the offspring were observed except for a slight (but statistically significant) decrease in fetal weights at 1,000 mg/kg/day. This finding was within the range of historical control data from the laboratory at which the study was conducted and was strongly influenced by the results of two litters. Based on these results, the authors concluded that 400 mg/kg/day AMPA was the NOAEL for both maternal and developmental toxicity.

Commercial herbicide formulation

One ~~non-compliant~~non-guideline developmental study was conducted using an unspecified commercial formulation of "Roundup", which was reported to consist of 360 g/l glyphosate and 18% (w/v) POEA (Dallegrave et al., 2003; Table 3). Sixty pregnant Wistar rats were divided into four treatment groups and dosed once daily by gavage with 0, 500, 750, or 1,000 mg/kg/day "glyphosate-Roundup" on GD 6-15. The treatment description provided was ambiguous as to whether the dosages stated were for "Round-up" or the active ingredient, glyphosate. Body weights were recorded daily, and food and water consumption recorded at appropriate intervals. On GD 21, animals were sacrificed and the effects of treatment on development of the resulting offspring were recorded. It is difficult to draw any conclusions regarding the developmental effects of Roundup from this study. Not only are the treatment doses unclear, but the number of animals per group is significantly lower than the recommended 25 rats/group, and the highest dose group was further reduced by ½ due to animal deaths. Furthermore, very few data are actually presented in the paper. Rather, maternal body weight is reported as relative weight, with weights on GD 0 considered 100%. Likewise, food and water intake are presented graphically as relative intakes, although it is not clear from the paper to what values these reported intakes are normalized. Similarly, fetal findings are presented as percentages or unsubstantiated means throughout the paper, which complicates their interpretation. When one converts these mean reproductive indices to actual totals, one discovers that the data presented are flawed (see Table 3). For example, an animal should have at least as many implantation sites as fetuses and the number of corpora lutea should be greater than (or at least never less than) the number of implantation sites; however, in the 500 mg/kg/day treatment group, more fetuses were reported than implantation sites. This brings into question the reported resorption rate for this treatment group. Also, in the 750 mg/kg/day treatment group, more implantation sites than corpora lutea are reported.

Comment [D12]: Tables 3-8 not presented

The authors report a dose-related increased incidence of skeletal alterations of 15.4, 33.1, 42.0, 57.3 % in the control, 500, 750, and 1,000 mg/kg/day treatment groups, respectively. The most frequently observed alterations include incomplete ossification of the skull and enlarged fontanels. Interestingly, examination of the

list of skeletal alterations observed in this study shows an extremely high prevalence of incomplete ossification of various bone structures. These findings are signs of a developmental delay that correct themselves within a brief period. It is important to note that the methods described to fix and stain the fetal skeletons for evaluation are unusual and it is possible that the method led to artifacts that were falsely categorized as alterations. The standard method calls for fetuses to be fixed in alcohol and macerated with potassium hydroxide before staining (Dawson, 1926; Wilson, 1965). In this study, however, fetuses were fixed in formalin, and later immersed in a solution of trypsin before staining with alizarin red. Since trypsin is a proteolytic enzyme, it is likely that it would have digested some of the peptide bonds of the bone matrix, resulting in areas that would appear as if they were incompletely ossified. Based on the use of these questionable methods, and the obviously flawed reporting of the data, it is impossible to draw any conclusions regarding the developmental effects of "Roundup" treatment from this paper. Furthermore, because a commercial formulation was used, it is not possible to attribute any observed effects to glyphosate, specifically.

Reproductive Studies

Glyphosate

~~Two~~ Three multi-generational reproductive studies tested glyphosate, two in Sprague Dawley rats (Schroeder, 1981, 1982; Reyna, 1990; ~~Table 4)~~ and one in wistar derived rats (Moxon, 2000 Table 4)). Although conducted prior to the establishment of GLP, the Schroeder study received a quality assurance audit by the testing facility and generally adhered to current testing guidelines. In this study, diets initially contained 0, 30, 100, and 300 ppm glyphosate and were adjusted weekly to maintain approximate glyphosate dose levels of 0, 3, 10, and 30 mg/kg/day throughout. The test substance was administered starting 63 days prior to mating of the first generation and continuously thereafter. Three generations of parents (F_0, F_1, and F_2) were raised to maturity and mated. Each generation was mated twice (24 females and 12 males per treatment group per mating) with a rest period of at least 14 days between weaning and mating, resulting in two sets of litters per generation. The first litter of each generation was sacrificed at weaning and necropsied. Offspring of the second litter were randomly selected for mating to produce the next generation for study. Histopathology was conducted on 10 males and 10 females from each control and high dose treatment group for each parental generation (F_0, F_1, and F_2) and offspring of generation F_{3b}. Clinical observation data, mean body weights and food consumption were comparable across control and treatment groups for all generations (data not shown). Mating, fertility and pregnancy indices showed considerable variability across the study, but no consistent dose-related trends were evident. Mean gestation length was comparable among control and treatment groups for each mating and in all generations, as were mean numbers of total, live, and dead pups per dam and the male/female sex ratios of pups. Pup weights throughout weaning and the mean number of pups weaned per litter were similar among control and treatment groups for each mating and all generations. Statistical differences in postnatal pup survival indices were noted between control and some treated groups in each

Comment [D13]: Tables 3-8 not presented

Comment [D14]: Tables 3-8 not presented

Comment [drf15]: Need to add Moxon data to the table

generation; however, no dose-related trends could be discerned. It should be noted that, in the second mating of the F_0 generation, reduced pup survival in the treated groups for postnatal days (PND) 4-21 was mainly attributed to high pup mortality in one or more litters at each treatment level; as such, differences in pup survival indices between control and treated groups were concluded to not indicate an adverse effect of treatment. Terminal body, organ, and organ/body relative weights were comparable across all control and treatment offspring of the F_0 and F_1 generations, and for males of the F_2 generation (data not shown). F_2 female offspring from the treatment groups, however, exhibited significantly lower liver/body weight ratios compared to controls, although no dose-related trend was apparent. Also, mean spleen weights were higher in the F_2 mid-dose females compared to controls, but the low- and high-dose weights were comparable to control values. Because a clear dose-response was not observed across the generations, these data were not considered indicative of a treatment-related adverse effect. An equivocal increase in tubular dilation of the kidney observed in the high-dose male F_{3b} pups was not considered to be related to treatment as it was not observed in a second study (Reyna, 1990; see below) conducted at much higher doses. Gross postmortem observations and histological evaluations of offspring from all generations also failed to demonstrate any treatment-related adverse effects. No NOAEL values were reported by the study authors.

The study by Reyna (1990) was done according to the previously established EPA guidelines, OPP 83-4. In this study, male and female Sprague Dawley rats (30/sex/group) were fed diets containing 0, 2,000, 10,000, or 30,000 ppm glyphosate starting approximately 11 weeks prior to mating. It is of note that the high dose exceeds by 50% the current limit dose for dietary studies (20,000 ppm). These diets continued to the end of study and throughout all generations. Litters of the first generation were culled to eight pups each on PND 4 and weaned on PND 21, at which time 30 rats/sex/group were randomly selected for creation of the F_2 generation. The selected animals were allowed a 14 week growth period before being mated twice, resulting in creation of the F_{2a} and F_{2b} generations. All animals were observed twice daily for mortality/morbidity, and body weights were recorded weekly. Food consumption was also recorded weekly up until mating, after which determinations were continued through gestation and lactation for females only. Weekly assessments for clinical signs of toxicity were also made on adults. Pup weights and signs of clinical toxicity were recorded on PND 0, 4, 14, and 21. All litters were culled to eight pups each on PND 4. F_0 and F_1 adults were examined by gross necropsy. Also, all culled pups, those that died postnatally, those not selected for mating, and all F_{2a} and F_{2b} weanlings were examined by gross necropsy. The F_0 and F_1 adult ovaries and testes (including epididymides) were weighed. Histopathology using H&E stain was conducted on all tissues retained from control and high dose treatment groups of the F_0 and F_1 generations and on one weaning/sex/litter from these treatment groups of the F_{2b} generation.

Comment [drf16]: Don't know why David puts in the guideline here but not for other studies ... either delete all or add to other studies

Clinical signs included soft stools, reduced food intake, and decreased body weights in male and female rats of both the F_0 and F_1 generations fed 30,000 ppm glyphosate. Mean body weights of high dose animals were

maintained at 8-11% below control throughout the study. Body weight gains during gestation, however, were comparable among females from the control and high dose groups. Body weight effects were not observed in the middle and low dose treatment groups. Glyphosate treatment had no effect on mating, pregnancy, or fertility indices. Gestational lengths were also unaffected. The mean number of pups per dam of the F_0 generation's high dose group was slightly (albeit not significantly) reduced compared to control. A similar, although less substantial, difference between control and high-dose animals was noted as a result of the first, but not the second, mating of the F_1 generation. Because the differences in litter size between the high dose and control groups were not statistically significant and not observed as a result of all matings, it is unlikely the effect was a result of treatment. The percentages of live and dead pups and the male/female sex ratios were similar across treatment groups for all generations. Mean pup weights at birth and initial weight gains were comparable across all treatment groups and generations. As animals reached the age of weaning (PND 21), however, weight gains for the high dose pups had significantly waned compared to controls for all generations. Researchers theorized that, as pups began supplementing their milk intake with consumption of the prepared diets towards the end of the lactation period, food intake of pups in the high dose groups likely lagged behind that of control animals. No gross or microscopic pathological changes related to glyphosate treatment were noted for adult animals or their offspring. The kidney effects noted in the Schroeder (1981,1982) study (which used lower doses than those in this study) were not confirmed. Based on these results, 10,000 ppm (~~approximately 500 mg/kg/day~~ 694 mg/kg/day) is considered the NOAEL for ~~maternal~~ paternal toxicity and 30,000 ppm (~~approximately 1500~~2132 mg/kg/day) is considered the NOAEL for reproductive and developmental toxicity.

Comment [drf17]: Or systemic?

The study by Moxon (2000) was compliant with the revised 1998 EPA guideline, OPPTS 970.3800. In this study male and female wistar derived rats (26/sex/group) were fed diets containing 0, 1000, 3000 and 10000 ppm glyphosate starting 10 weeks before mating. These diets continued to the end of study and throughout all generations. Twenty six males and twenty six females per groups were selected from F1A litters to become F1 adults. F1A litters were separated from the mothers on *post partum* day 29. After at least 10 weeks the mating regimen was followed until F2A litters were produced and weaned to day 29 *post partum*. All males were terminated following littering and females terminated on or soon after day 29 of lactation. Body weights of males and females were measured weekly during the pre-mating periods and female body weights were measured on days 1, 5, 8, 15 and 22 of gestation and days 1, 5, 8, 15, 22 and 29 *post partum* (where day 1 *post partum* was the day of birth).

Comment [drf18]: Again make them consistent or delete

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No adverse effects of glyphosate were noted on reproductive performance and the number of successful matings was similar for all groups. No effect of glyphosate was noted on pup survival, litter size during lactation, clinical condition of pups or the ratio of male to female pups in either F1A of F2A litters. No effect of glyphosate was noted on pup birth weight for weither F1A or F2A pups. Body weights of F1A pups in the high

dose group were lower as compared to controls at day 29 post partum, but this effect was not observed for the F2A pups. F1 males selected for mating had a subsequent reduction in body weight during the pre-mating period. No effects were noted on time to pre-putial separation or day of vaginal opening. No effects were noted for the number of sperm, sperm motility parameters or sperm morphology for either F0 or F1 males. No effect of glyphosate on organ weights in F0 and F1 parents were noted. No effects were seen in high dose females for the number of primordial and small growing follicles (F1, left ovary). No macroscopic or microscopic findings were attributable to glyphosate in any tissue from F0 or F1 animals. No effects of glyphosate on brain, spleen or thymus weights and no macroscopic findings were observed in any tissue of the F1A or F2A pups. Fertility and reproductive performance of each generation of parental animals and clinical condition and survival of offspring were not adversely affected. The NOAEL for ~~this study~~reproductive and parental toxicity was 3000 ppm (323 mg/kg/day) ~~was~~ based on reductions in body weight and feed consumption in the F1 males at 10000 ppm (1105 mg/kg/day)~~mean NOAEL doses were: 282.6 mg/kg/day and 322.8 mg/kg/day during 10 weeks pre-mating for F0 male and female parents respectively; 351.8 mg/kg/day and 370.8 mg/kg/day during 10 weeks pre-mating for F1 male and female parents respectively; 271.3 mg/kg/day and 284 mg/kg/day during gestation for F0 and F1 parents respectively; 724.1 mg/kg/day and 780.6 mg/kg/day during lactation for F0 and F1 parents respectively).~~

Comment [drf19]: I added the overall mean dose for males and females for the F0 and F1 and then divided by 4 ... sure there are other ways to do this ...

Comment [drf20]: I don't have a copy of the mouse study but I would simplify this section to match the other repro studies. What does the study have for offspring/developmental NOAEL?

POEAs

Two reproductive/developmental screening studies were conducted with two POEA surfactants covering a range of carbon chain lengths and polyalkoxylation. ~~Knapp (2007, 2008) evaluated two POEA surfactants in reproductive/developmental screening studies.~~

In (Knapp, 2007), ~~a~~In the first study (Knapp, 2007)groups of male and female ~~Charles River Sprague~~Sue-Dawley rats (20/sex/group) were ~~dosed~~treated with a ~~tallow derived~~ POEA incorporated into the diet at 0, 100, 300 and 1000 ppm.—Reproductive endpoints for evaluation included gonadal function, mating behavior, conception, parturition and lactation of the F0 and F1 generations and development of the F1 and F2 generations through postnatal days 70 (F1 animals not selected for breeding phase) and 4 (F2). F0 animals ~~were approximately 10 weeks of age at study start, with continuous dosing started 70 days before mating to fully evaluated sperm cycle.~~ Survival and clinical condition, reproductive performance, body weight and food consumption (pre-mating, gestation and lactation), organ weights, and macroscopic and microscopic morphology of the F0 and F1 parental generations; developmental landmarks, estrous cyclicity, spermatogenic endpoints and testosterone and thyroid hormone levels of the F1 generation; the clinical condition and body weight of the F1 and F2 litters; and litter viability and postnatal survival of the F2 litters were unaffected by test material administration at all concentrations in the F0 and F1 generations. Litter loss, increased mean number of unaccounted-for sites and decreased mean number of pups born, live litter size and postnatal survival from birth to postnatal day 4 occurred in the 1000 ppm group following breeding of the F0 females. These differences were not always statistically significant compared to the control group and the effects were not reproduced following breeding of the 1000 ppm group F1 animals. The equivocal changes observed following breeding of the 1000 ppm group were not considered a clear signal of an effect but precluded consideration of the 1000 ppm group as a clear NOAEL for F0 reproductive and F1 developmental/neonatal toxicity. However, based on the results of this screening study, the a dose level of 300 ppm (16.6 and 14.9 mg/kg/day for the F0 and F1 males, respectively, and 19.5 and 18.9 mg/kg/day for the F0 and F1 females, respectively17.5 mg/kg/day) was considered to be a clear NOAEL ~~(no observed adverse effect level)~~ for F0 reproductive toxicity and F1 developmental/neonatal toxicity, of tallow derived POEA when administered continuously in the diet to rats.

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Comment [drf21]: Inserted 3 different write ups for this study

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Comment [D22]: Taken directly from the revised report summary conclusion, substituting MON 0818 with "tallow derived POEA "and MON8109 with "coco derived POEA"

Knapp (2008) conducted a second reproductive/developmental screening study using the OECD 422 test guideline with a different POEA surfactant at 0, 30, 100, 300 and 2000 ppm in the diet of Sprague-Dawley rats (12/sex/group). In addition, the POEA surfactant from the 2007 study was reevaluated at the dietary concentration of 1000 ppm to determine whether the equivocal litter effects at the high dose were repeatable. Systemic parental toxicity was exhibited at 2000 ppm as mean body weight losses, lower mean body weight gains and food consumption for both sexes. Lower mean live litter size on postnatal day 0, number of pups born and implantation sites, lower mean pup weights on postnatal day 1 and reduced pup survival were noted in the 2000 ppm group. Therefore, the NOAEL was considered to be 300 ppm (23 mg/kg/day). Regarding the repeat study with the first POEA surfactant no test substance-related signs of systemic toxicity, reproductive effects or effects on pup survival or morphology were noted at 1000 ppm. The NOAEL for this POEA was considered to be 1000 ppm (81 mg/kg/day).

Comment [D23]: Taken from the report summary conclusion

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SEE SUMMARY DOCUMENT FOR THESE POEA REPRO STUDIES SENT SEPERATELY

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Reproductive/Developmental Data from Other Animal Studies

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Glyphosate

The National Toxicology Program (NTP) conducted a 13-week glyphosate feeding study in F344/N rats and B6C3F$_1$ mice (Chan and Mahler, 1992). Groups of ten male and ten female rats and mice were administered feed containing 0, 3,125, 6,250, 12,500, 25,000, and 50,000 ppm glyphosate for 13 weeks. An additional ten animals per sex and species were included at each dose level for evaluation of hematological and clinical pathology parameters. At the end of study, necropsies were conducted on all animals. For the screening of potential reproductive toxicity, epididymal tail, epididymal body, and testicular weights, sperm motility, sperm counts, and testicular spermatid head counts were evaluated for male rats and mice in the control and three highest dose groups (12,500, 25,000, and 50,000 ppm glyphosate). Similarly, vaginal cytology and estrous cycle lengths were evaluated for female rats and mice from the same dose groups. Glyphosate treatment did not affect survival of either rats or mice, but did reduce terminal body weights of male rats and mice in the 25,000 and 50,000 ppm treatment groups (Table 5). ~~Body~~ Mean body weights of female ~~rats and~~ mice in the highest glyphosate treatment group (50,000 ppm) ~~were~~ was also marginally affected (data not shown). The weights of the left testis as well as the cauda and corpus of the left epididymis were not affected by glyphosate treatment of rats or mice. Similarly, no effects of treatment on sperm motility, spermatid counts, and spermatid head counts were observed in either species. A statistically significant decrease in concentration of spermatozoa in fluid withdrawn from the caudal epididymis compared to controls was observed in male rats treated at the two highest glyphosate concentrations. Also, rat estrous cycle length among animals exposed to 50,000 ppm glyphosate was longer than that of controls, which is consistent with the reported weight loss. Similar effects on spermatozoa concentrations and estrous cycle lengths were not observed in treated mice. The biological significance of these findings is not clear; however, in the opinion of the study authors, these findings were not considered evidence of adverse effects on the reproductive system.

Yousef et al. (1995) investigated the effects of subchronic glyphosate treatment on semen characteristics in New Zealand white rabbits (Table 6). The study consisted of a six-week preliminary period in which no

treatment was provided, followed by a six-week treatment period, and finally, a six-week recovery period in which treatment was discontinued. Groups of four glyphosate-treated rabbits received either 1/100 LD_{50} (low dose) or 1/10 LD_{50} (high dose) administered orally in a gelatin capsule. The exact doses of glyphosate administered cannot be determined from the study report because neither the LD_{50} value from which the doses were determined, nor the study from which the LD_{50} value was obtained, was reported. Additionally, it is not clear if animals were dosed daily or weekly, or whether an herbicide formulation or pure glyphosate was used. On a weekly basis, body weights were recorded and ejaculates were obtained using a teaser doe. The following parameters were measured for each ejaculate sample: volume, sperm concentration, percentages of dead and abnormal sperm, methylene blue reduction times (MBRT; an indicator of sperm quality), initial fructolytic activity (an indicator of sperm vitality), and sperm osmolality. In general, body weights and all sperm parameters appeared to be adversely affected by treatment and showed some improvement during the recovery period. Based on these results, one cannot conclude, however, that glyphosate treatment induced a harmful effect on semen quality. The dosages and frequency of exposure are unknown. Furthermore, the methods do not indicate whether control animals were sham-handled during the treatment period. If they were not sham-handled, then the effects of treatment may be stress-related. Based on the authors' own statistical analyses, they were unable to detect a dose-response relationship for any of the measured parameters, with the exception of dead sperm per ejaculate during the recovery period. The lack of an overall dose-response further suggests that the observed results are random, rather than a direct effect of glyphosate treatment. Additionally, the rabbits used in the study were quite small (~3 kg); buck rabbits used for mating purposes are usually larger, often weighing 4-5 kg. This suggests the animals may not have been full mature. Based on the aforementioned shortcomings (failure to report numerical data, lack of detail in methods description; group sizes that were too small; and absence of a dose-response), it is impossible to draw conclusions regarding the effects of glyphosate treatment on male rabbit fertility.

Commercial herbicide formulations NEED TO ADD THE ROMANO 2010 STUDY REVIEW

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Dallegrave et al. (2007) conducted a ~~non-compliant~~non-guideline developmental-reproductive study using a commercial formulation of Roundup (exact formulation unspecified) reported to contain 360 g/l glyphosate and 18% (w/v) POEA (Tables 7 and 8). Sixty pregnant female Wistar rats (15/group) were gavaged daily with 0, 50, 150, or 450 mg/kg/day "glyphosate-Round-up" throughout pregnancy and lactation. As previously discussed in reference to an earlier study by the same group of researchers (Dallegrave et al., 2003), it is not clear whether the stated doses are of glyphosate or the Roundup formulation. Maternal body weights were recorded daily during pregnancy. Litter size, the numbers of living, dead, and viable pups, and the sex ratio of the offspring were recorded at birth. From the end of lactation until puberty, pup weights were recorded weekly and both general and sexual development of the offspring followed. One male and one female per litter were sacrificed at puberty (65 days of age) as well as at the age of adulthood (140 days of age) to investigate systemic and reproductive effects of treatment. For the females, sacrifices occurred on the first estrus after 65 or 140 days of

age. Organ weights were recorded relative to total body weights. Additional investigations were also conducted on the males. The numbers of homogenization-resistant spermatid per testis and spermatozoa per epididymis tail were counted using a hemocytometer. Daily sperm production was determined by dividing the number of spermatid per animal by 6.1 days and the epididymal sperm transit rate was calculated by dividing the number of epididymal sperm by the daily sperm production rate. Sperm morphology was assessed via microscopic examination of 200 sperm rinsed from the deferens ducts. Histological examination of the testes was conducted on five testes per treatment group to assess mean tubule diameter, the number of tubules with elongated spermatids, and the general condition of the testicular tissues. Finally, blood testosterone levels were determined by radioimmunoassay.

At the doses administered in this study, maternal toxicity was not observed and reproductive outcome data (number of pups, sex ratio, etc.) and pup weights were unaffected by treatment. A non-dose related delay in vaginal opening in females and early preputial separation in the high dose males were noted; however, these findings were all within the normal physiological range for the species and in line with historical control data. No other effects on the female offspring were observed. Male offspring at puberty exhibited a statistically increased percentage of abnormal sperm at the low, but not the medium or high dose, suggesting a random finding. A dose-related decrease in blood testosterone levels was also observed at puberty, with the finding at the high dose being significantly different from control. Interestingly, this result is contrary to what would be expected if the early preputial separation was a true finding. Additionally, the effect on testosterone levels was no longer evident at the age of adulthood. Also in adulthood, daily sperm production and sperm number were significantly reduced in the low and high dose groups, but not the medium dose group compared to controls; thus, no dose-related findings were observed. Finally, upon histological examination of the testis, the authors noted a reduction in elongated spermatids and the presence of vacuolization at puberty and degeneration of the tubular lumen at adulthood in some of the animals in the treated groups. Unfortunately, the micrographs provided in the study report are at too low of a magnification and too small to draw conclusions; however, other findings are evident in the micrographs – specifically, enlarged interstitial cells – that the authors fail to mention in their report. This obvious omission suggests that the authors have limited experience doing these types of histological examinations and that the findings may be an artifact of processing rather than a true effect of exposure. Additionally, none of the guideline-compliant reproductive studies nor the NTP 13-week subchronic study discussed previously – all of which involved much greater glyphosate exposures – reported such testicular anomalies.

Two ~~non-compliant~~non-guideline studies have been conducted using Herbicygon, a commercial herbicide formulation containing glyphosate; no other formulation information provided (Daruich et al., 2001; Beuret et al., 2004). In both studies, the activity levels for certain enzymes in the liver (as well as in the heart and brain in Daruich et al. [2001]) were measured in both dams exposed to Herbicygon via drinking water and their fetuses.

Female Wistar rats were mated and then divided into control and treatment groups, with eight rats per group. Exposures began on GD 1 and were continued throughout gestation to GD 21. Control animals received tap water. In Daruich et al. (2001), treated animals received drinking water with 0.5% or 1% "glyphosate solution (w/v)"; in Beuret et al. (2004), treated animals received 1% "glyphosate solution (w/v)". Despite the assertion that animals were dosed with glyphosate, the test solutions of drinking water were most likely prepared with Herbicygon. Because the glyphosate concentration of Herbicygon is not provided, it is impossible to know exactly how much glyphosate treated rats received in these experiments. Additionally, Herbicygon is a commercial herbicide formulation, and as such, most likely contains a surfactant. Thus, any effects noted in these studies cannot be definitively attributed to glyphosate, the surfactant, or a combination of the two ingredients.

In Dariuch et al. (2001), body weights, food intake and water consumption were measured daily. After two weeks of treatment, it was noted that treated rats had reduced their food and water intake compared to controls. In an attempt to account for the possible effects of restricted diet on the study results, a fourth treatment group of six rats was added to the study. This low diet group did not receive Herbicygon treatment, but was provided with minimal food and water (10 g of rat chow and 10 ml drinking water daily, administered sometime after a period of regular food and water consumption). On GD 21, maternal and fetal livers, hearts, and brains were removed, washed, and stored at -20°C for subsequent analysis. Fetal organs were pooled. Tissues were homogenized, centrifuged to obtain the cytosolic fraction, and analyzed to measure the enzymatic activities of isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, and malic dehydrogenase. Herbicygon-treated dams consumed significantly less food and water, and gained significantly less weight than controls (mean weight gains of 80.70 and 52.79 grams in the 0.5% and 1% treatment groups, respectively, versus 92 grams in the control group). Maternal liver (but not heart and brain) weights were also significantly decreased. Animals in the restricted diet group also gained significantly less weight during gestation than controls (49.51 grams versus 92 grams in the controls) and displayed statistically smaller livers than control animals. These results suggest that the effect of treatment on body and organ weights may be due to reduced food and water intakes rather than a direct effect of Herbicygon treatment. It is difficult to draw any further conclusions from this paper. Although various statistical increases and decreases in enzymatic activity of maternal and fetal organs were noted (data were provided graphically in original study and are not reproduced here), a consistent effect of treatment was not observed and dose-response relationships were generally lacking. Additionally, only the cytosolic fractions from organ homogenates were evaluated. Thus, the information gathered may be misleading because the enzymes monitored are found in both the cytosol and mitochondria. For example, in rat brain, only 45% of malic enzyme activity is in the cytosolic isoform (Vogel et al. 1998). Furthermore, despite the inclusion of a low diet control group, a possible effect of diet restriction on enzymatic activity cannot be completely ruled out for the Herbicygon-treated animals. Many researchers have found that food restriction (in the absence of toxicants) affects the activity of many enzymes, including those

examined in this study (Sassoon et al., 1968; Nagy et al., 1978; Sachan and Das, 1982; Martins et al., 1985, 1986; Martin et al, 1990; Xie et al., 1995; Boll et al., 1996; Goodridge et al., 1996, 1998). Daruich et al. do not state exactly when the low diet treatment group began receiving its restricted diet, and the average food and water consumption data presented in the study report suggest that this may not have occurred until as late as half-way through gestation; thus, although the data show that the period of restricted diet was sufficient to affect total maternal weight gain and liver weights, it may not have been long enough to affect changes in organ enzyme activities. In order to appropriately control for reductions in food and water consumption in the herbicygon treatment groups, pair-fed control animals should have been included. Thus, based on the use of inappropriate controls, treatment with a commercial herbicide rather than pure glyphosate, unknown exposure levels, and a lack of consistent dose-response data, conclusions regarding the results of Daruich et al. (2001) cannot be made.

Dosing in the second study using Herbicygon (Beuret et al., 2004) was as discussed above. It should be noted that the study authors incorrectly referred to glyphosate as an organophosphate ~~pesticde~~pesticide. In this study, body weights, food intake and water consumption were measured daily. On GD21, serum samples were taken from dams for measurement of lipid peroxidation using a thiobarbituric acid reactive substances (TBARS) assay. Maternal and fetal livers were homogenized and assays were conducted to measure levels of lipid peroxidation products, and glutathione peroxidase, catalase, and superoxide dismutase activities. Although not specifically stated in the methods section, it appears that fetal livers from each litter were pooled for analyses. Treated dams consumed significantly less food and water, and gained significantly less weight during gestation than controls (mean weight gain of 53 grams versus 92 grams for controls). As well, liver weights were reduced in treated rats compared to controls. Despite these maternal differences, average fetal body and liver weights did not appear to be affected by treatment. Because the number of fetuses per litter is not provided in the study report, it is not known if the reduced body weight gain in the dams affected the number of fetuses surviving to term. Serum lipid peroxidation levels of the dams were not affected by treatment. Lipid peroxidation levels in the livers of treated dams and their fetuses, however, were increased over that of controls (dams: 1.6 ± 0.05 µg TMP/g tissue versus 0.9 ± 0.02 in the controls; fetuses: 9.8 ± 2.7 versus 2.4 ± 0.9). Glutathione peroxidase activity levels were also increased with treatment in the fetuses (13.28 ± 0.58 µmol NADPH/min/g tissue versus 9.03 ± 1.01 in the controls), but not in the dams. Liver catalase and superoxide dismutase activity levels were not affected by treatment. It is impossible to draw any conclusions regarding the effects of Herbicygon treatment from these data because no restricted diet controls were included in the study. Other research has shown that dietary restriction can affect lipid peroxidation and glutathione peroxidase activity levels (Rao et al., 1990; Kim et al., 1996; Mura et al., 1996); therefore, it is not known if the effects observed resulted from treatment or reduced food and water intake. Furthermore, even if the effects observed in this study were directly related to treatment, whether they were due to glyphosate or other agents included in the Herbicygon formulation (including ~~the~~ a surfactant) can not be determined. In

summary, because of inadequate information regarding dosing, limited sample numbers, and the lack of appropriate controls, no conclusions can be made regarding the effects of glyphosate on liver enzyme activity in treated dams and their offspring.

Conclusions – Developmental and Reproduction Studies

Based on a review of the available developmental and reproductive studies, no data exist from studies that have been conducted using good laboratory practice (GLP) protocols and/or according to established testing guidelines to indicate ~~that prenatal exposure to~~that glyphosate, POEA surfactants, or commercial glyphosate herbicides are developmental or reproductive toxicants. ~~formulations are associated with adverse developmental effects in animals~~. While a few studies have claimed ~~that~~ reproductive or developmental effects ~~are associated with exposure~~ (Dallegrave et al., 2003; Yousef et al., 1995; Dariuch et al., 2001; Beuret et al., 2004; Romano et al., 2010), these studies suffer from numerous inadequacies in design and reporting. Many of these studies appear to have used commercial herbicide formulations rather than pure glyphosate or surfactant, have not included appropriate controls, have used inadequate numbers of animals per treatment group, and did not clearly state doses or dose rates. Furthermore, no consistent dose-related trends in effects were observed in these studies. The studies with glyphosate that have been conducted using ~~good laboratory practice~~ (GLP) protocols and/or according to established testing guidelines have found no effects of treatment on reproduction or in offspring, despite significant toxicity in treated dams (IRDC, 1980b; Holson, 1990, 1991; Schroeder, 1981, Reyna, 1990). The exception is a single rat teratology study that found an increase in resorptions, a decrease in the number of fetuses per dam, and reduced fetal weights associated with gavage administration of 3,500 mg/kg/day glyphosate on GD 6-19 (IRDC, 1980a). These effects, however, were associated with significant maternal toxicity, including death in 6/25 rats treated at this dose. It is important to note that the current limit dose for oral gavage studies is 1,000 mg/kg/day for studies required by regulatory agencies. ~~These effects, however, were associated with significant maternal toxicity, including death in 6/25 rats treated at this dose~~. In conclusion, the animal data, as a whole, indicate that glyphosate is not a selective developmental or reproductive toxicant.

Mechanistic Studies

A number of studies have been performed to investigate the potential impact of glyphosate and glyphosate-based herbicides on a variety of biological processes. The vast majority of these studies have used *in vitro* models or non-mammalian *in vivo* models, such as the sea urchin. When possible, toxicity data derived using the same model system, but different types of test substances (pure glyphosate versus glyphosate-based formulations), are highlighted. These data provide an indication of the relative impacts of glyphosate versus

other formulation additives on the observed effect(s). The studies presented here are categorized according to the biological processes examined. Emphasis is placed on those processes that could contribute to developmental and reproductive perturbations, although some of the information provided also relates to general mechanisms of toxicity.

Developmental/Reproductive Toxicity Studies

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Benachour et al., (2007) assessed the *in vitro* toxicity of four glyphosate-based formulations, glyphosate, a POEA surfactant and AMPA on HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. Cells were exposed in serum-free medium for 24 hour to a number of concentrations for each test material. Cell viability was measured using the MTT assay. This assay measures the reduction of MTT (a yellow tetrazolium salt) to blue-colored product (formazan) in living cells. The Toxilight bioassay was used to assess cell membrane damage. Adenylate kinase (AK) is released into culture medium when cells dies. AK actively phosphorylates ADP to form ATP which can then be measured using the bioluminescent firefly luciferase reaction. The Caspase-Glo™ 3/7 Assay was used to screen for apoptosis. Activation of the caspase cascade is an important event in the apoptotic pathway.

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Pagenelli

Genotoxicity/Clastogenicity Studies

Numerous studies have investigated whether glyphosate is genotoxic (acts directly with DNA to cause genetic mutation) or clastogenic (causes direct damage to whole chromosomes). Due to the extensive nature of this database, an in-depth review of these studies is well beyond the scope of this paper; however, an excellent weight-of-evidence assessment of the genetic toxicology for glyphosate, some glyphosate-based formulations and the major formulation surfactant, POEA, can be found in Williams et al. (2000), and the reader is directed to this reference for a more detailed discussion. In short, Williams et al. (2000) concludes that the evidence as a whole indicates that glyphosate does not pose a genotoxic risk for humans. A similar conclusion was drawn by the Food and Agricultural Organization of the United Nations (FAO, 2005). In making this assessment, emphasis was placed on the possible carcinogenic and/or tumorigenic potential of glyphosate; however, because heritable changes to the genetic material of germ cells could be passed onto the developing offspring, this assessment is also pertinent to a discussion of possible developmental effects related to exposure. In the interest of brevity, only those genotoxicity studies conducted since the publication of Williams et al. (2000) are reviewed here. These are also tabulated in Table 9.

Comment [D24]: While this is an accurate reference for the summary of FAO/WHO determinations, the more detailed "Toxicological Evaluations document (2006) from the same 2004 meeting in Rome has more detail on specific expert review & opinions.

In Chruścielska et al. (2000), an Ames test and *in vivo* micronucleus assay were conducted on glyphosate and Perzocyd 10 SL, a commercial glyphosate-based formulation. The Ames test is a common assay for genotoxicity. Conducted with and without the addition of metabolic enzymes, this assay determines a test

article's ability to induce mutation in bacteria and whether metabolism plays a role in a substance's genotoxicity. The micronucleus assay, a common test for clastogenicity, looks for the prevalence of polychromatic erythrocytes in rodent bone marrow. Some of these cells will contain micronuclei, small particles of chromosomal material that "lag behind" in the anaphase stage of cell division. Micronuclei can be observed under normal conditions, but are more frequently seen after exposure to clastogenic substances. The Ames test was conducted in TA 97a, TA 98, TA 100, and TA 102 strains of *Salmonella typhimurium*, with and without S9 activation, using concentrations of glyphosate that ranged from 0.6-1.2 mg/plate (three plates/concentration). Although the authors state that the Ames test was negative, the results are somewhat confusing because it is unclear from the paper whether glyphosate, Perzocyd 10 SL, or both were tested in the assay. This study would be improved by including additional species of bacteria in the Ames test (especially *E. coli* WPA, which increases the assay's sensitivity by providing an additional bacterial organism and target gene).

In the micronucleus assay, groups of six 7-12 week old mice, weighing approximately 30 grams each, were administered an intraperitoneal (IP) injection of either water (negative control), 75 mg/kg Endoxan (positive control), 300 mg/kg glyphosate, or 90 mg/kg Perzocyd 10 SL. The concentration of Perzocyd 10 SL used in this study was alleged to be 70% the median lethal dose (LD_{50}). Animals were examined at 24, 48, and 72 hours following administration. For each animal, 1,000 polychromatic cells were examined for the presence of micronuclei, indicative of chromosomal damage. No statistically significant differences between treated and negative control animals were observed. Although the study would be improved by the use larger numbers of mice per group in the micronucleus assay, these assays appear to have been generally well-conducted.

Monroy et al. (2005) examined the cytotoxicity and genotoxicity of glyphosate in GM38, a human primary fibroblast cell line, and HT1080, a human fibrosarcoma cell line. To assess chronic toxicity, 100 µl of a 3×10^3 cells/ml suspension were treated with 0.9 – 8.5 mM (GM38 cells) or 0.6 – 3.3 mM (HT1080 cells) glyphosate at 37°C for 72 hours in a total volume of 200 µl. Following incubation, the cells were washed and cell viability measured using crystal violet (sic) staining. To determine acute toxicity, 100 µl of a 3×10^5 cells/ml suspension were first allowed to adhere to the microtiter plates over a 24 hour period before they were washed and then treated with 4.0 – 6.5 mM glyphosate at 37°C for four hours in a total volume of 25 µl. Following incubation, cells were trypsinized, then treated with trypan blue to assess cell viability. Cell culture medium was used as a negative control for both assays; VP16 was used as a positive control in the acute assay only. Under chronic conditions, 80% cell viability was measured at 0.6 and 5.5 mM glyphosate in the HT1080 and GM38 cells, respectively. Cell viability of only 50% was seen at 1.7 and 6.9 mM glyphosate in the same respective cell lines. Under acute conditions, viability of 80% or greater was seen at glyphosate concentrations of 5.5 and 6.5 mM in the HT1080 and GM38 cells, respectively. Interestingly, these concentrations are much lower than those that were reported to be neither genotoxic nor cytotoxic in human fibroblast cells (Leuken et al., 2004;

discussed below). To evaluate genotoxicity, the Comet assay was conducted on both cell lines following four hour pretreatment with 4.0 – 6.5 mM glyphosate. Briefly, cells were mixed with agarose on a microscope slide, lysed with trypsin, electrophoresed, and 25 cells per treatment group observed via microscopy. Both the average distance of migration of the comet tail as well as the degree of cell damage (based on a five class scale) were assessed visually; statistical analyses were done on the migration distances only. The authors reported that the average DNA migration distance was increased over control values at glyphosate concentrations ≥4 mM and ≥4.75 mM in the GM38 and HT1080 cells, respectively. Unfortunately, the results of these experiments are difficult to interpret because the authors fail to mention whether they adjusted the pH of the 25 mM glyphosate working solutions used in the experiments. Certainly, treatment of cells with an acidic pH has been shown to cause DNA damage to cells (Xiao et al., 2003). Thus, it is possible that the effects observed in this study could have been mediated by the pH of the treatment solution. Furthermore, various software packages are commonly used for the automated scoring of Comet assay parameters, including mean tail length. Because this parameter is not typically scored visually (Collins, 2004), it is not known if a manual measurement of this parameter is reliable. Finally, mean tail length has been reported to increase only while comet tails are first being established (i.e., at relatively low initial damage levels, Collins, 2004), and thus, may not be an appropriate measure of DNA damage.

Çavaş and Könen (2007) treated goldfish with Roundup in their tanks at concentrations equal to 5, 10, or 15 ppm glyphosate. The negative control was no treatment; the positive control was 5 mg/l cyclophosphamide. Blood samples were taken for genotoxic analysis from the caudal vein of specimens (5 per dose per duration group) on the 2nd, 4th, and 6th days of treatment. For the micronucleus assay, five slides per fish were prepared and stained with 10% Giemsa solution. A total of 1,500 peripheral erythrocytes per slide were scored manually for micronuclei; nuclear abnormalities other than micronuclei were also assessed. The Comet assay was conducted using standard methods. Two hundred cells per slide and five slides per fish were scored manually under 400× magnification. Both the mean percentage of cells with DNA damage as well as an index of genetic damage (based on classification of the cells on a 0-4 scale) were calculated for each specimen. At two days treatment, the incidences of micronuclei and total nuclear abnormalities were increased at 15 ppm only. At four and six days treatment, the incidences of both were statistically increased in all dose-groups and in dose-dependent manners. Additionally, the index of genetic damage was statistically increased in both a dose- and duration-specific manner over that of control. Because the commercial formulation was used in these experiments, however, it cannot be said whether the observed responses were due to the active ingredient, glyphosate, or some other component of the formulation, such as the surfactant. It also is not clear if the culture medium pH was adjusted upon treatment.

Poletta et al. (2009) examined the potential genotoxicity of Roundup in caimans treated *in ovo*. Eggs collected within five days of oviposition were each treated with 50-1750 µg Roundup (Full II formulation with 66.2%

glyphosate; 10-12 eggs per group taken from multiple clutches). As a positive control, some eggs were treated with 700 or 1400 µg cyclophosphamide per egg. Following hatching, peripheral blood samples were collected from the spinal vein of the animals. For the micronucleus assay, two blood smears per animal were stained with acridine orange and 1,000 erythrocytes each were manually scored from two replicate slides. The Comet assay was conducted using standard methods; one hundred cells per animal (50 each per replicate slide) were scored manually on a 0-4 scale and a DNA damage index calculated. The high dose of cyclophosphamide induced 90% embryo mortality and extensive malformations in the single surviving caiman. Additionally, the low dose of cyclophosphamide resulted in malformations of the digits in two of the 22 caimans treated *in ovo*. No malformations were observed in any of the Roundup-treated groups. Both genotoxicity assays showed a significant increase in DNA damage following cyclophosphamide treatment as well as in the groups treated with 500 µg or more of Roundup. Again, as in Çavaş and Könen (2007), the cause of the observed response cannot be determined because the commercial formulation (including surfactants and excipients) was used instead of glyphosate alone.

Dimitrov et al. (2006) examined three different herbicides, including Roundup, for their abilities to induce chromosomal aberrations and micronuclei in a plant (*Crepis capillaries L.*) and a mouse bone marrow test system. The plant assays were run using concentrations of herbicide bracketing those typically used in agricultural practice; in the case of Roundup, these concentrations ranged from 0.05-1.0%. Both a negative (distilled water) and positive (ethyleneimine) control were also evaluated. Following a 2 hr treatment and a 1 hr wash, plant tissues were colchicine-treated and fixed. To evaluate chromosomal aberrations, 400 cells and 50 metaphases per slide were analyzed; to evaluate for micronuclei, 4,000 cells and 1,000 interphase cells per treatment were assessed. The mammalian assays were done using C57BL mice (8 per treatment group). Chromosomal aberrations were assessed using bone marrow cells from mice treated orally for 6-120 hrs with Roundup equal to ½ the LD_{50} (as determined in a preliminary study). Colchicine (4 mg/kg ip) was administered following treatment and 1-1.5 hrs prior to bone marrow extraction and processing. From each animal, 50 metaphase cells were evaluated for chromosomal abberrations, for a total of 400 cells per treatment. Analysis of micronuclei was done using polychromatic erythrocytes from mice treated orally for 6-120 hrs with Roundup equal to 1/8, ¼, or ½ the LD_{50}. Both a negative (distilled water) and positive (100 mg/kg cyclophosphamide) control were also evaluated. Following extraction and processing, 500 cells per animal, for a total of 4,000 cells per treatment group were evaluated for micronuclei. None of the Roundup concentrations induced chromosomal aberrations in the plant or mouse bone marrow cells. Also, Roundup treatment did not increase the number of micronuclei in mouse polychromatic cells. While some of the Roundup treatments induced a slight increase in micronuclei in the plant assay, these increases were neither dose-dependent nor statistically significant.

Lueken et al. (2004) studied the synergistic impact of peroxide-induced oxidative stress with chemicals considered by the authors to be nongenotoxic. Genotoxicity was assessed using the comet assay following the same general procedure as that in Monroy et al. (2005), described above. Cytotoxicity was measured using the MTT assay, which assesses enzymatic activity by measuring the conversion of a yellow tetrazolium salt to a blue, soluble formazan (Hansen et al., 1989). Production of the formazan product is indicative of sufficient mitochondrial activity typical of healthy cells. Based on results from the comet assay and MTT assay, only concentrations of test chemicals that were both nongenotoxic and noncytotoxic were used in the study. For glyphosate, this concentration was reported to be > 75 mM (exact concentration not indicated). Peroxide, at 40 and 50 µM, in combination with > 75 mM glyphosate, induced a synergistic increase in genetic damage to fibroblasts. This increase was not as marked as that observed with the other peroxide-chemical combinations tested (4-chloroaniline, 2,3,4,6-tetraclorophenol, *m*-xylene, and *n*-hexanol), and this may be why the effect of glyphosate was not fully discussed in the study report. Although it is difficult to glean much information from these results because the exact concentration of glyphosate used was not specified, it is noteworthy that concentrations less than 75mM were neither cytotoxic nor genotoxic. Further, this finding is in stark contrast with Monroy et al. (2005, described above) who observed cytotoxicity at 1.7 and 6.9 mM glyphosate.

Piešová (2004) focused on micronuclei formation in bovine lymphocytes as a result of exposure to a formulation containing 62% glyphosate, and 38% inert ingredients by weight. Because the goal of this evaluation was to assess potential toxicity to livestock from glyphosate exposure, bovine peripheral lymphocytes, rather than a more commonly used Chinese Hamster Ovary (CHO) or human peripheral lymphocyte cell types, were used. Whole blood bovine lymphocytes were derived from two donor bulls. The glyphosate formulation was diluted in sterile water to concentrations of 28, 56, 140, 280, and 560 µmol/l prior to treatment of the lymphocyte cultures. Mitomycin C (0.4 µM) was used is the positive control because it is known to be genotoxic and to increase micronuclei formation (Moore et a., 1995); a negative control was also included, likely consisting of distilled water, though the composition of this control was not explicitly stated in the manuscript. The cultures were incubated (likely at 37°C, though this was not specified) for 24 or 48 hours. At least 1,000 bi-nucleated lymphocytes were scored for the presence of micronuclei. No increase in micronucleus frequency was observed after the 24 hour incubation at any concentration. An increase in micronucleus frequency was reported at 48 hours at the 280 µmol/l, but not at 560 µmol/l, for one donor's cells. Using the second donor's cells, an increase was seen at 48 h at 560 µmol/l only. Overall, this study indicates that concentrations of the glyphosate formulation >280 µmol/l are potentially linked to an increase in genotoxicity; however, whether this effect is due to glyphosate or another formulation ingredient in unclear.

In a similar study by Šiviková and Dianovský (2006), chromosomal aberrations (CA) and sister chromatid exchanges (SCE) were assessed following *in vitro* herbicide treatment of bovine peripheral lymphocytes. As was the case in Piešová (2004), the specific name of the herbicide formulation used in this study was not

provided, but it is stated to consist of 62% glyphosate and 38% inert ingredients. Also, bovine lymphocytes were derived from two donor bulls, as in the previous study, largely to address the potential for toxicity to livestock. For the CA assay, bovine peripheral lymphocytes were incubated in 28, 56, 140, 280, 560, and 1120 µmol/l concentrations of glyphosate (as the herbicide formulation) for 24 hours. Ethyl methanesulfonate was used as a positive control; the composition of the negative control was not specified. At least 1,000 metaphases were analyzed per sample, and the mitotic index was determined for 2,000 cells per sample. No statistically significant increase in the number of CA was observed at any of the glyphosate concentrations tested. For SCE assessment, cells were incubated for 24, 48, or 72 hours using the same glyphosate concentrations as in the CA assay, with mitomycin C as the positive control. Fifty metaphase stages per sample were assessed, and the mitotic index was derived from an examination of 100 metaphases per sample. At 24 hours, the number of SCE/cell was increased at glyphosate concentrations $\geq$56 µM, and an increase in the proliferation index was seen at 560 and 1120 µmol/l. After 48 hours, an increase in SCE was seen at 280 and 560 µmol/l in one donor and at 560 µM in the other. The frequency of SCE in cells treated with 1120 µmol/l was not determined because of cell death. After 72 hours, only concentrations $\leq$ 280 µmol/l were tested, due to cell death at greater glyphosate concentrations. In both donors, an increase in SCE was observed at 140 µmol/l, but not at 280 µmol/l. Although the authors concluded that glyphosate was able to induce SCE, and at higher concentrations, could inhibit cell cycle progression in bovine lymphocytes, such a definitive statement cannot be made. Because a glyphosate-containing herbicide formulation was used in these experiments, the extent to which glyphosate, versus other formulation ingredients, was responsible for the observed effects cannot be determined. Furthermore, the study authors do not state whether they adjusted the pH or osmolarity of the cell media upon addition of the test agent, which is important becasue evidence suggests that such factors can affect the frequency of SCE (Galloway et al., 1987). Finally, it is not clear to what extent cytotoxicity interfered with the test results.

In Kaya et al. (2000), the *Drosophila melanogaster* wing spot test was used to assess the genotoxicity of glyphosate and three additional pesticides. This assay utilizes two crosses of Drosophila: a standard cross and a high biotactivation cross. Drosophila eggs from these crosses were allowed to hatch and the larvae were collected 72 ± 4 hr later and placed in nutritive medium prepared with test articles dissolved in either distilled water or 5% acetone. Concentrations of pure glyphosate ranging from 0.1 to 10 mM in distilled water were used. The positive control contained 1 mM of the known mutagen ethyl methanesulfonate. The larvae were exposed until pupation, then collected and stored in 70% ethanol. Afterwards, wings were removed, mounted, and examined under 400X magnification for the presence of cell clones (spots) showing malformed wing hairs. With the standard cross, glyphosate induced a weak, but significant, increase in the frequency of small single spots and total spots at concentrations of 2, 5, and 10 mM. No increases in wing spot frequency were observed in the high bioactivation cross, indicating that metabolism reduced the genotoxic potential. Whether the P450 activity in humans is more appropriately represented by the standard or the high biotransformation

cross is unclear; thus, although an increase in mutagenicity was seen in the standard cross, more investigation is needed to extrapolate these results to the human.

~~Finally, i~~In a study by Mañas et al. (2008), the potential genotoxicity of AMPA, the environmental breakdown product of glyphosate and phosphonic acids (such as EDTMP and DTPMP) in detergents (Skark et al., 1998), was assessed. Hep-2 cells were treated with 2.5-10.00 mM AMPA in culture for 4 hrs. Both a positive control (0.01 mM mitomycin C) and negative control (MEM media alone) were also included. Following processing, 100 "nucleoids" per treatment were evaluated for percent tail DNA, tail length, and tail moment (percent tail DNA × tail length) using the Comet Score 1.5 software package. AMPA treatment induced a dose-dependent increase in all three parameters compared to control. In another experiment, human lymphocytes from six donors were treated in culture to 0.9 and 1.8 mM AMPA for 48 hr. Two thousand cells per treatment were then examined for mitotic index and 100 metaphases analyzed for chromosomal aberrations. Again, mitomycin C (0.9 µM) was used as a positive control. In this case, 1.8 mM AMPA, but not 0.9 M AMPA, induced a statistically significant increase in the number of chromosomal aberrations. Finally, Balb-c mice were treated with two ip injections of either 100 or 200 mg/kg AMPA each (5 animals per group; treatments separated by 24 hrs). The positive control group received 20 mg/kg cyclophosphamide; the negative control group received saline injections. Twenty-four hours after the final treatment, the animals were sacrificed and bone marrow smears prepared for analysis of micronuclei (1,000 erythrocytes per animal). Additionally, the ratio of polychromatic-to-normochromatic erythrocytes was evaluated for 500 cells per treatment. Although AMPA treatment statistically increased the number of micronuclei per 1,000 cells analyzed, this increase did not appear to be dose-dependent. The interpretation of these study findings is difficult. Treatment of cells with an acidic pH has been shown to cause DNA damage to cells (Xiao et al., 2003), and the authors do not mention whether they adjusted the pH of the culture media upon AMPA treatment. Furthermore, the AMPA concentrations and *in vivo* doses used in these experiments are extremely high compared to what one might reasonably expect to encounter in the environment. For example, Williams et al. (2000) estimate worst case aggregate AMPA exposures for female applicators and children of 4.8-10.4 µg/kg/d – a level of exposure 1,000-2,000-fold lower than that administered to the mice in this study.

Finally, in a study by Gasiner et al., (2009). The Coment assays was used evaluate the potential for glyphosate to damage DNA in individual cells. HepG2 cells exposure to a glyphosate-base formulation (Grands Travaux® 400 g/L of glyphosate – R400) during 24 h at different concentrations ranging from 1–10 ppm of the formulated product. Benzo[a]pyrene (50

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M, B[a]P) was used as positive control. All experiments were repeated 3 times in duplicate for 100 cells. Following processing the reading of the slideswas performed with a fluorescence microscope (40×). Nuclei observed were classified into 4 classes: 0 (undamaged), 1 (minimum damage), 2 (medium) and 3 (maximum damage).The comet assay measures DNA strand breaks as evidenced by enhanced migration of DNA from nuclear bodies in gel electrophoretic conditions. It is very clear that this endpoint is not uniquely responsive to agents that interact with DNA to cause strand breaks. A number of studies have shown that positive

responses can be observed in the comet assay that are mediated by toxicity rather than direct DNA interactions. Examples include positive comet responses due to apoptosis and necrosis induced by hyperthermia (Fairbairn et al., 1996), hypoxic cytotoxicity (Brezden et al., 1997), cytokine treatment (Delaney et al., 1997), oxidative damage (Collins et al., 1997) and exercise in human volunteers (Hartmann et al., 1994). Other studies have demonstrated that mutagenicity and comet responses are not necessarily closely related (Speit et al., 1996). It is clear that results from this assay should be carefully interpreted with respect to the mechanism involved. DNA damage was observed only at concentrations that resulted in significant cytotoxicity (75% DNA damage in the treatment group compared to 35% DNA damage for the controls was reported for HepG2 cells exposed for 24 hours to the R400 formulation). However, Table 1 shows that the 50% effect level for HepG2 cell viability, as measured with the alamar blue test, was 12 ppm. Consequently, DNA damage was being assessed in a HepG2 population that experienced 50% loss of viability, which invalidates the results and conclusions from this assay. The way the data from comet assays is presented is not standard. Data from this assay is normally presented as median tail % + SD, as it is presented in paper there is no indication of the variation within the data nor is there any statistical analysis to substantiate their claims. In addition, some of the buffers used in the comet assay are also poorly defined e.g the electrophoresis buffer which is just stated as pH13 buffer (this is a normal pH for this buffer but clearly it would be useful to know what it is), the unwinding time used was 40 min rather than the more standard 20 min and they don't quote the electrophoresis conditions in terms of volts/cm which is typical to do. Manual counting of comet slides is obviously very heavily influenced by the operator and hence is very outdated. Currently, image analysis software is readily available which helps remove operator bias/judgement effects. The strong level of negative evidence from multiple assays that are directly or closely linked to mutagenic endpoints reinforces the conclusion that the comet findings observed are not associated with interaction of glyphosate or other formulation components with DNA, but they are secondary to effects such as toxicity (Williams et al., 2000).

Summary - Genotoxicity/Clastogenicity Studies

As a whole, the results of these studies provide weak evidence that glyphosate formulations increase genotoxicity, and even weaker evidence that glyphosate itself, in the absence of other substances, has any impact on this endpoint. Most studies did not specifically report the concentration of glyphosate used in the experiment. Further complicating the matter is that the results from some experimental models (*e.g.*, the Drosophila model) are difficult to extrapolate to the human and some studies report somewhat contradictory results. Overall, the genotoxicity/clastogenicity studies published since the Williams et al. (2000) review have not provided information that strongly supports adverse effects associated with glyphosate exposure.

Cell Cycle/Transcriptional Inhibition Studies

Several studies have been performed to ascertain whether glyphosate is likely to inhibit cell cycle progression and transcription (Table 10). These studies have been performed using the sea urchin (*Lytechinus variegatus*) model, which is often used to assess aquatic toxicity, but is of questionable value in gauging human health risk. Additionally, the majority of these studies use herbicide formulations containing glyphosate as the test article, rather than glyphosate alone.

Medina et al. (1994) first examined the impact of a formulation identified as "Roundup" on the sea urchin. In this manuscript, the authors discussed the advantages of the use of the sea urchin as a biomarker of toxicity; particularly, its sensitivity to a variety of compounds and the ease of handling the model. Sea urchin embryos were exposed to 480 g/L "Roundup" (with a final concentration of 1.4×10^{-4} M glyphosate) three minutes after appearance of the fertilization membrane. It should be noted that this concentration of glyphosate is 34x greater than the allowable maximum contaminant level of glyphosate in drinking water (US EPA, 2009). The investigators observed that the Roundup-treated eggs exhibited deformed or destroyed nuclear elements, as well as a perforated nuclear membrane. It is noted however, 1) that Roundup formulations contain surfactants; 2) that the observations are consistent with the effects of a surfactant; and 3) that the impact of glyphosate alone was never assessed.

In Marc et al. (2002), the impacts of glyphosate alone and a formulation identified as "Roundup" (containing 170 g/L of isopropyline glyphosate salt) on the cell cycle was examined. Concentrations of Roundup <1.0% were not lethal to the urchin embryos; however, treatment with concentrations ≥0.8% (a concentration much higher than what would be used for herbicidal purposes) led to a delay in the time to M phase entry in the first cell division following fertilization. It was also reported that, although pure glyphosate ≤ 25 mM had no effect on cell division, adding progressively larger amounts of glyphosate to 0.2% Roundup (which already contains 2 mM glyphosate) increased the delay to cell division. The data to support these claims, however, are very weak. The figure meant to illustrate the increase in cell cycle delay upon addition of glyphosate to Roundup shows that, when no glyphosate was added to the Roundup, there was no delay in the first cell division – a direct contradiction to the authors' previous claims. Furthermore, when glyphosate was added at increasing concentrations, no dose-response relationship was evident. Finally, no statistics were shown, suggesting that the significance of the glyphosate-induced effects was not tested. Thus, the claim that glyphosate potentiates the action of Roundup on cell division is not supported by the data. Because CDK1/cyclin B is an important modulator of progression into the M phase of mitosis, the investigators next assessed the kinetics of CDK/cyclin B activation using histone H1 as a substrate for phosphorylation. Roundup was found to inhibit CDK/cyclin B activation and to reduce protein production, as indicated by a methionine incorporation radioassay. The amounts and phosphorylation status of cyclin B were also determined by Western blot analysis of whole embryo extracts; however, no clear differences between untreated and Roundup-treated cells were observed. Based on these results, no conclusions can be made regarding the effects of glyphosate on cell division. In addition, the study had several design flaws. For instance, appropriate controls were not included in these experiments. Given that cell division is highly affected by pH, temperature and ionic concentration, a relatively non-toxic solution with these same characteristics should have been used as the negative control. Furthermore, glyphosate alone was not examined. Rather, evaluations involved the herbicidal formulation Roundup, which contains surfactants having the potential to affect cell division.

The impact of a formulation identified as Roundup on cell division and activation of CDK1/cyclin B was further investigated in Marc et al. (2003). The authors again demonstrated that Roundup slows first cell divisions in the sea urchin when applied after fertilization. The impact of CDK activity in treated and untreated sea urchin embryos was measured by affinity-purifying CDK1/cyclin B at selected times following fertilization (≤ 120 minutes), and determining the kinase activity of the enzyme complex using H1 as a substrate for phosphorylation. The results from this experiment are difficult to interpret. Although the authors claim that Roundup treatment reduced CDK1/cyclin B complex activity, the figure presenting these findings shows only one-sided upper value standard error (SE) values for the control group, and no SE bars for the point of maximum CDK1/cyclin B activation in the controls. Also, although the sea urchin embryos treated with Roundup were said not to undergo CDK1/cyclin B activation, enough activation was obviously present to induce cell division, albeit delayed. Additionally, using an assay to look at protein synthesis via incorporation of radiolabeled methionine, Roundup appeared to decrease protein production during the first two hours, which could potentially inhibit or delay various reproduction processes. In the last set of data presented, the authors looked at cyclin B abundance and phosphorylation status at 60 and 75 minutes after fertilization using an antibody detection method. As shown in their previous paper (Marc et al., 2002), Roundup did not affect CDK1/cyclin B activation. Overall, the data presented in this paper do not clarify whether the delayed phosphorylation of cyclin B observed following Roundup treatment is due to the delay in cell division or vice versa. Furthermore, because an herbicidal formulation was used in these experiments, no conclusions can be made regarding the potential actions of glyphosate alone on cell cycle division.

In Marc et al. (2004a), the effects of a variety of glyphosate-based herbicides on cell cycle progression in the sea urchin embryo were investigated. Herbicides assayed included Roundup 3plus, Amega, Cargly, Cosmic, and Roundup Biovert. The percentage of embryos undergoing the first post-fertilization cell division was assessed by phase microscopy at 60-minute intervals up to 300 minutes post-fertilization. All herbicides tested inhibited cell cycle progression; however, the effects observed were not proportional to the glyphosate content of the herbicides. When tested at equivalent glyphosate concentrations, Amega, Cosmic and Cargly were all more effective than Roundup 3plus and Roundup Biovert in delaying the first cell division. These results suggest that a formulation ingredient other than glyphosate may be mediating this effect. Cytological observations revealed no aberrant chromosome morphology in relation to the delay in cell cycle progression for any of the compounds tested.

Marc et al. (2004b) then went on to examine whether Roundup 3plus inhibits CDK1/cyclin B activation by preventing dephosphorylation of the complex at tyrosine 15. Sea urchin cells were treated with Roundup 3plus at a concentration equivalent to 10 mM glyphosate, after which CDK1/cyclin B complex was affinity-purified from embryo extracts at 10-minute intervals post-fertilization. Following extraction from the beads, the affinity-purified proteins were resolved by gel electrophoresis. Using western blot analysis, cyclin B and CDK1 protein

expression were assessed, as well as the tyrosine phosphorylation of CDK1. For these experiments, CDK1 abundance was deemed to be not affected by treatment, and was therefore used as a gel-loading control; however, CDK1's expression against that of another protein that is more commonly used for such purposes was never evaluated. Considering that these experiments were designed to look at effects on CDK1/cyclin B activation, the use of CDK1 expression as a loading control seems highly inappropriate. From this experiment, the authors report that herbicide treatment delays the cyclin B pattern changes associated with activation of CDK1 during the first post-fertilization cell division. Furthermore, treatment delayed CDK1 tyrosine phosphorylation by 30 minutes compared to control and this delay corresponded with the delay in CDK1/cyclin B activation. To evaluate whether the delay in phosphorylation was due to an effect of treatment on phosphatase activity, the effects of both 10 mM glyphosate and Roundup 3plus (at a concentration equivalent to 10 mM glyphosate) on the phosphatase activity of recombinant cdc25C protein and embryo extracts were assessed. Neither of the treatments induced changes in the phosphatase activity of recombinant cdc25C or embryo extracts. Next, DNA synthesis, as measured by the incorporation of radiolabeled thymidine, was assessed at various times post-fertilization. During the first cell division, herbicide treatment inhibited DNA synthesis by approximately 70% compared to control. From these results, it is not clear how herbicide treatment may mediate an inhibition of DNA synthesis or how such an effect may translate to a delay in CDK1 tyrosine phosphorylation. Furthermore, the fact that the authors did not present data using pure glyphosate in the DNA synthesis experiment is interesting, especially since they purportedly used glyphosate alone in the phosphatase assays. It appears likely that the observed effects on DNA synthesis are not mediated by glyphosate, but rather, by another component of the Roundup 3plus formulation.

In the final paper by Marc et al. (2005), the authors examined the effect of various glyphosate formulations on transcription and sea urchin hatching kinetics. For most experiments, Roundup 3plus was used; however,other formulations including Cargly, Cosmic, and Roundup Biovert were also tested in some assays. Hatching was observed with phase contrast microscopy and expression of sea urchin hatching enzyme mRNA (SgHE) was measured by RT-PCR. Transcriptional activity was quantified by incorporation of 5-[^{3}H] uridine in a sea urchin embryo suspension. Actinomycin D, a known transcription inhibitor, was used as a positive control. In the first experiment measuring the effect of Roundup 3plus on hatching kinetics, sea urchin embryos at the morula stage (after 4-6 cycles of cell division) were exposed to 2, 4, and 6 mM of Roundup (30 replicates per concentration). The morula stage was chosen because previous studies showed that Roundup slowed the first cell divisions (Marc et al., 2002, 2003), and the authors wished to focus on the impact of Roundup on later cell divisions and transcription. Interestingly, the positive control agent was applied 10 minutes following fertilization rather than at the morula stage. Why the test agent and positive control were not applied at the same developmental stage or what the effects may be of application at different stages is not known. A dose-related decrease in the percentage of embryos hatching after Roundup 3plus treatment was observed. The authors also measured the delay in hatching time due to administration of 8 mM pure glyphosate, 0.2% Roundup, and

0.2% Roundup supplemented with 8 mM glyphosate. Test agents were again administered during the morula stage (four trials/treatment group). Pure glyphosate delayed hatching by 33 ± 6 min, 0.2% Roundup resulted in a 128 ± 30 minute inhibition, and the 0.2% Roundup plus 8 mM glyphosate supplementation resulted in a delay of 205 ± 30 minutes. Thus, although glyphosate alone had little effect, co-administration of additional glyphosate with Roundup increased hatching delay time. The authors interpreted these results to mean that the surfactant included in the Roundup formulation was not solely responsible for Roundup's effect on hatching time; however, a statistical analysis of the results was not conducted, and only four replicates were run per treatment group. Interestingly, in another experiment, pure glyphosate tested at concentrations of ≤ 8 mM has no effect on hatching time. The reasons for the discrepancy between these results and those of the previous experiment are not clear. The four glyphosate-containing formulations (Roundup Biovert, Roundup 3plus, Cargly, and Cosmic), however, all caused delays in hatching, although some formulations were more potent than others. Despite the lack of effect with neat glyphosate, the authors concluded that glyphosate must be detrimental because all four of the formulation products led to hatching time delays. In a final experiment, it was reported that Roundup 3plus applied at the morula stage of sea urchin development decreased transcription, as indicated by a decrease in 5-[^{3}H] uridine incorporation. Also, SgHE mRNA expression was reduced for two hours post-fertilization; however, the results of only a single experiment were shown to support this claim and no statistical analyses were conducted. Overall, these experiments show that glyphosate-based herbicidal formulations impact cell divisions in the sea urchin embryo; however, they do not provide evidence that glyphosate is the cause of these effects. In fact, these studies indicate that glyphosate itself is significantly less toxic to sea urchin embryos than the commercial herbicidal products, suggesting that the observed effects are due to another component of the formulations.

A study by Amouroux et al. (1999), which did not address glyphosate, but rather examined the toxicity of three commonly-used mild surfactants, ~~further~~ This results of this study provide support ~~suggests~~ that the effects observed in sea urchin embryos following herbicide application are due to a component of the formulations rather than glyphosate itself. In this study, the effects of cocamido propyl hydroxyl sultaine (CAS), magnesium laureth sulfate (Mg LES), and decyl glucoside (APG) on inhibition of egg cleavage, calcium homeostasis, intracellular pH, sodium and potassium contents, protein and DNA synthesis, and protein phosphorylation were measured. All the surfactants tested caused inhibition of cleavage at doses lower than those commonly used in consumer products. Additionally, both CAS and Mg LES induced changes in membrane permeability and ionic disequilibrium. APG was found to alter intracellular pH and decrease DNA synthesis. Although POEA, the surfactant used in Roundup and many of the other commercially available glyphosate-based herbicides, was not specifically examined in this study, these findings suggest that toxicity to sea urchin eggs appears to be a common feature of surfactants. Thus, the findings of similar toxicity upon application of herbidical formulations containing similar surfactants should be considered unremarkable.

Summary - Cell Cycle/Transcriptional Inhibition Studies

Overall, results using the sea urchin model have shown that exposure to high concentrations of glyphosate-based herbicide formulations and substances used on consumer products can lead to cell cycle delay. Despite these findings, the relevance of such studies for the human health risk assessment of glyphosate is questionable. The relationship between cell division in sea urchin eggs directly exposed to high concentrations of pesticides versus the effects in humans exposed dermally or orally to much lower concentrations of glyphosate-based herbicides is tenuous at best. Generally, concentrations ≥ 8 mM glyphosate were used in these studies (Medina, 1994; Marc et al., 2002, 2003, 2004a, b, 2005). This concentration equates to an average body burden of 1.8 g isopropylamine glyphosate/kg body weight. For a 55 kg person, this would be equal to 100 g glyphosate, or the amount that would be found in 0.6 L of Roundup, if it were to be directly ingested (Kutzman and DeSesso, 2003). Additionally, the majority of experiments addressing the impact of glyphosate in the sea urchin model were conducted using Roundup-branded or other herbicide formulations, rather than neat glyphosate. Evidence that glyphosate, and not the surfactants present in these formulations, was involved in the observed effects is sorely lacking. Finally, there is no strong evidence to support the notion of Marc et al. (2003) that glyphosate potentiates the toxicity of Roundup branded herbicides.

Endocrine Disruption

In recent years, many environmental pollutants have been suspected to contribute to endocrine disruption; however, only a few have been scientifically proven to disrupt the endocrine system at environmentally relevant concentrations (WHO, 2002). Mechanistic studies to ascertain whether glyphosate can cause adverse developmental or reproductive effects by interfering with the functioning of the endocrine system have been conducted (Table 11). These studies are varied in their approach and examine potential effects on steroid hormone production and placental enzyme activity. In a number of cases, glyphosate-based formulations containing surfactant systems were evaluated for aromatase activity using microsomes. These studies are flawed from the outset because microsomes are denatured by very low concentrations of surfactants and detergents. This is noted in the *US EPA Endocrine Disruptor Screening Progam Test Guideline OPPTS 890.1200: Aromatase (Human Recombinant)*, which clearly warns that all glassware and apparatus used in the microsome preparations should be free of detergent residue. Furthermore, if detergent residues compromise study viability, testing measurable concentrations of detergent like substances would overload such *in vitro* systems and is certainly not a viable approach to investigating endocrine disruption. Levine et al (2007) evaluated a variety of surfactants using an *in vitro* systems and determined results were due to ~~the~~ a non-endocrine mechanism of compromised mitochondrial membrane potential and altered permeability of cell membranes.

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Petit et al. (1997) screened various herbicides, fungicides, insecticides, xenobiotics, and phytoestrogens for estrogenic potency using two *in vitro* systems: a recombinant yeast system expressing the rainbow trout estrogen receptor and rainbow trout hepatocyte cultures. Yeast cells containing a *lacZ* reporter gene linked to two estrogen-responsive elements were treated in culture at 10^{-8} to 10^{-4} M of each test agent for four hours. 17 β-Estradiol was used as the positive control. β-Galactosidase activity, dependent on expression of the *lacZ* gene, was measured in Miller units using a colorimetric substrate. To ensure that the absence of a response was not due to toxicity, cell density measurements were made before and after treatment, although the data for agents that were not estrogenic were not shown. Glyphosate treatment had no effect on the basal level of β-galactosidase activity. Only those test agents shown to be positive for estrogenicity in the yeast system, plus eleven other randomly selected test compounds, were evaluated in the trout hepatocyte cultures for expression of the vitellogenin gene, as determined by slot blot analysis; glyphosate was not among those tested. One weakness of this study is that the description of methods is not clear as to whether pure glyphosate or a glyphosate-based herbicide was tested. Nevertheless, these data provide no evidence of estrogenic activity.

Lin and Garry (2000) investigated whether certain herbicides and fungicides commonly used in the Red River Valley of Minnesota could induce proliferation of the estrogen-responsive MCF-7 cell line. MCF-7 cells were seeded in media containing either regular fetal bovine serum (FBS) or steroid growth-factor-deficient FBS (produced through prior treatment with 10% charcoal dextran). Following a 48-hour incubation, the cells were then treated with different dilutions of test chemicals, 10^{-9} M estradiol (positive control), or solvent vehicle (negative control). After seven days in culture, cell numbers and viability of harvested cells were assessed using a fluorescence-activated cell sorter. In separate experiments, cytotoxicity (following 72-hour incubation of MCF-7 cells in various concentrations of test agents) and apoptosis (using propidium iodide staining) were evaluated by flow cytometry. Both the "Roundup" branded formulation (identified as containing 0.99% glyphosate) and its active ingredient, glyphosate, were shown to induce proliferation of MCF-7 cells. This occurred in media containing either regular or steroid growth-factor-deficient FBS, suggesting that the proliferative effect was mediated through a nonestrogenic pathway. Maximum induction levels ranged from 121 ± 10.3% for 10 µg/ml "Roundup" in regular FBS and 135 ± 3.5% for 2.28×10^{-4} M glyphosate in steroid growth-factor-deficient FBS. None of the test agents used in these experiments was shown to be cytotoxic at the concentrations used in the seven-day proliferation studies. Also, neither glyphosate nor "Roundup" was shown to induce apoptosis. While these results suggest that glyphosate may be able to induce cell proliferation, this response is not mediated through an estrogenic pathway.

Comment [drf25]: This suggest this was a L&G product... maybe a good idea to go back and check all ref. for how they report the test materials and use the terminology used by the authors.

Using an *in vitro* system, Meulenberg (2002) tested the ability of various endogenous steroids, pharmaceutical agents, pesticides, and pollutants to displace estradiol (E_2) from human sex hormone-binding globulin (SHBG), a high affinity, but low capacity, hormone-binding protein found in the blood that functions in the transport of sex hormones and protects against their degradation. Changes in the binding capacity of SHBG will affect the

free concentrations of various sex hormones. Because it is assumed that only the free fraction of such hormones can exert biological activity, such changes will likely result in hormonally-mediated changes in the organism. Microtiter plates were coated with rabbit anti-SHBG antibody, and using these plates, SHBG was isolated overnight from the serum of pregnant women. Following several washes, tritiated E_2, along with the test compound, was added to the microtiter plates. Following 48 hours incubation, supernatant was removed from the plates and the amount of radioactivity in the media was measured using a scintillation counter. Because testosterone is known to have a three times greater affinity for SHBG than E_2, it was used as a positive control. The binding of varying concentrations of test agents was referenced to the standard curve for testosterone. Affinity of these compounds for SHBG was defined as an ability to displace tritiated E_2 to an extent comparable to that of testosterone. The study authors indicate that glyphosate showed ambiguous results for displacement of E_2 from SHBG, although actual experimental data were not shown. These results indicate that glyphosate should not affect the ability of SHBG to bind sex hormones in the blood.

In Xie et al. (2005), the estrogenic potency of glyphosate, three non-glyphosate-based herbicides, and two types of ethyoxylate-containing surfactants (R-11 and Target Prospreader Activator [TPA]) was determined using the *in vivo* rainbow trout vitellogenin assay. In fish, adult female production of vitellogenin is mediated by estrogenic activity; thus, vitellogenin expression is thought to serve as a biomarker for chemicals likely to alter estrogenic activity in fish and other animals. In this study, exposure of the fish for seven days to 0.11 mg/L glyphosate had no effect on vitellogenin levels, suggesting that glyphosate is unlikely to alter estrogenic activity. Recommended concentrations of both 2,4 dichlorophenoxyacetic acid and trichlopyr increased vitellogenin production, suggesting that these chemicals can exert estrogenic activity. Mixtures of both these pesticides with the surfactants led to a significant increase in vitellogenin levels.

Kojima et al. (2004) tested over 200 pesticides for their ability to act as agonists and antagonists to two human estrogen receptor (hER) subtypes, hERα and hERβ, and a human androgen receptor (hAR). For each hormone receptor of interest, Chinese hamster ovary cells were transfected with the appropriate cDNA expression vector, along with a reporter plasmid containing either an estrogen-responsive element or an androgen-responsive element, and a *Renilla* luciferase expression vector (used as an internal control for determining transfection efficiency). After three hours transfection, cells were dosed for 24 hours with varying concentrations of test agent. To assess antagonistic activity to hERα, hERβ, and hAR, test agents were co-administered to the appropriate transfected cells with either 10^{-11} M E_2, 10^{-10} M E_2, or 10^{-10} M 5α-dihydroxytestosterone (DHT), respectively. Following incubation, expression of the response element-linked luciferase reporter was measured and normalized against that of the *Renilla* transfection control vector. Agonist activity was measured as the concentration showing 20% relative effective activity (REC_{20}) as 10^{-10} M E_2, 10^{-9} M E_2, and 10^{-9} M DHT at the hERα, hERβ, and hAR, respectively. Antagonist activity was expressed as the 20% relative inhibitory concentration (RIC_{20}); that is, the concentration of test agent causing 20%

inhibition of activity of 10^{-11} M E_2, 10^{-10} M E_2, or 10^{-10} M at the hERα, hERβ, and hAR, respectively. Although not completely clear from the methods section of the paper, it appears that the authors deemed a test agent positive for agonist or antagonist activity when, at the range of concentrations tested (10^{-5} to 10^{-8} M), the test agent showed greater activity than the REC_{20} or RIC_{20}, respectively. The values presented in the paper are the mean and standard deviations derived from at least three independent experiments. Although glyphosate was tested, it was not identified as a chemical having agonist or antagonist activity at any of the three receptor sites evaluated. It must be noted that specific tests for cell toxicity were not conducted, although assays were conducted at concentrations ≤ 10^{-5} M to minimize cytotoxicity. Based on these results, glyphosate does not appear to affect hormone binding at the hERα, hERβ, or hAR.

In Walsh et al. (2000), researchers assessed whether glyphosate or Roundup could affect the synthesis of the steroidogenic acute regulatory (StAR) protein. The StAR protein, located on the outer mitochondrial membrane, transports cholesterol to the inner mitochondrial membranes (Granot et al., 2002). The study authors hypothesized that this protein might be particularly sensitive to environmental toxicants in general because its active precursor form is both highly labile and critically dependent on trophic hormone stimulation. Translocation of cholesterol across the mitochondrial membranes is a rate-limiting step in steroidogenesis, so slight disruptions of StAR function and/or synthesis can potentially cause adverse effects. In this study, the authors showed that Roundup (180 g/L glyphosate) significantly inhibited steroidogenesis (as seen by decreased progesterone production in MA-10 cells) by inhibiting StAR protein expression. The authors note that glyphosate alone, however, did not have any effect on steroidogenesis or protein production at any concentration tested (0-100 µg/mL), indicating that the effect on StAR was dependent on other components of the herbicide formulation.

Levine et al. (2007) investigated the potential role of the surfactant in a Roundup-branded formulation in the inhibition of progesterone production upon treatment of MA-10 mouse Leydig cells. In this study, MA-10 cells were exposed for two hours to various surfactants (LAS D-40 [a linear alkylbenzene sulfonate], alcohol ethoxylate, lauryl sulfate [SDS], and benzalkonium chloride), as well as a concentrated Roundup-branded Lawn and Garden herbicide (with 180 g/L glyphosate isopropylamine, and 6.53 g/L surfactant [primarily POEA]), and Roundup blank (formulation without glyphosate). Both the Roundup-branded formulation and Roundup blank decreased the hCG-stimulated increase in progesterone production. In both cases, the median inhibition concentration (IC_{50}) was approximately 5 mg/mL. IC_{50} values for the four other surfactants were similar to that of the Roundup branded formulation and Roundup blank, indicating that: 1) the effect on progesterone is largely attributable to the surfactant, and not glyphosate; and 2) surfactants, in general, decrease hCG-stimulated progesterone production. The impact of the various surfactants on StAR protein levels was also assessed by Western Blot analysis on hCG-stimulated and non-stimulated MA-10 cells. Exposure to the surfactants, Roundup-branded formulation, and Roundup blank resulted in decreased levels of

the 30 kDa form of StAR protein, but not the 37 kDa precursor form. Because formation of the 30 kDa form requires mitochondrial import and processing of the 37 kDa precursor, the effect of treatment on mitochondrial potential, an indicator of proper mitrochondrial membrane function, was measured using the JC-1 cationic dye. Treated MA-10 cells demonstrated a loss of normal mitochondrial membrane potential, meaning that proper import and processing of the 37 kDa form of the StAR protein was disrupted upon treatment. This finding explains the previously observed decrease in the 30 kDa form of the StAR protein. Additionally, this effect on mitochondrial membrane potential was made for benzalkonium chloride and the alcohol ethoxylate surfactants, the Roundup branded formulation, and Roundup blank at concentrations below those that affect steroidogenesis. Overall, these results strongly support the concept that the adverse effects of Roundup branded herbicidal formulations on steroidogenesis are not mediated by glyphosate exposure, but rather, by the effect of surfactants on unprotected cells in culture.

Richard et al. (2005) examined aromatase activity and mRNA levels in JEG3 cells (derived from a human placental choriocarcinoma cell line) exposed to pure glyphosate or unspecified Roundup. Because glyphosate affects the cytochrome P450 activity of plants (Lamb et al., 1998), the study authors hypothesized that mammalian aromatase (also a cytochrome P450 enzyme) could be adversely affected. Additionally, the authors wished to further investigate claims made in other studies that glyphosate and/or an unspecified Roundup branded formulation cause reproductive/ developmental problems. The "Roundup" formulation was diluted in water to concentrations of ≤2% based on the recommended concentration for agricultural use of 1-2% in water. Concentrations of pure glyphosate equivalent to those present in the range of Roundup dilutions tested were also used. Aromatase activity was measured at one and 18 hours post treatment by determining the amount of tritiated water released from the radiolabeled aromatase substrate, [1β-^{3}H]-androstendione. RT-PCR to amplify aromatase and GAPDH (as an endogenous control) mRNA was performed. General cell viability was also measured. Roundup had a more pronounced effect on cell viability than equivalent concentrations of pure glyphosate, indicating that the formulation ingredients played an important role in cytotoxicity, as discussed previously for in vitro systems where surfactants are added. Pure glyphosate did not affect aromatase activity at one or 18 hours at any concentration tested (≤ 0.8%, or the highest dose at which marked cytotoxicity was not observed). Likewise, aromatase mRNA levels were unaffected by 18 hour treatment with ≤0.1% glyphosate. Incubation of the cells in Roundup for one hour, however, increased aromatase activity at all concentrations examined (0.02-0.2%). In contrast, incubation in Roundup for 18 hours caused a dose-dependent decrease in aromatase activity at all doses tested (≤0.8%). Levels of aromatase mRNA were also significantly decreased upon 18 hour incubation with 0.02 and 0.06% concentrations of Roundup. It was noted that, if glyphosate was combined with 0.02% Roundup, a greater decrease in aromatase activity after 18 hour incubation was seen than with 0.02% Roundup alone; however, the concentration of pure glyphosate used in this experiment was not indicated. The authors also measured aromatase activity in microsomes prepared from human full-term placental tissues incubated for 15 minutes

with higher concentrations of Roundup and glyphosate (≤10 and 1.1%, respectively). In this case, Roundup and glyphosate significantly decreased aromatase activity at concentrations of >0.05% and ≥0.5%, respectively. Because significant cytotoxicity would not be expected at 15 minutes post treatment, the decrease in aromatase activity likely is not due to cell death. Based on additional experiments using microsomes derived from equine testis, the study authors conclude that the rapid decrease in microsomal aromatase activity is due to competitive inhibition; however, only data using Roundup are presented in the paper. Based on these results, the authors conclude that the additives in Roundup play a key role in its effect on aromatase, but that glyphosate itself can elicit toxic effects as well. Although it was shown that pure glyphosate added to Roundup further decreased aromatase activity, the concentration of glyphosate required to elicit this effect was not indicated. Finally, in interpreting such findings for human health risk assessment, one must consider that the internal glyphosate concentration anticipated to reach sensitive tissues is several orders of magnitude lower than those used in this study. Because these experiments were all conducted in an unvalidated *in vitro* system using physiologically irrelevant concentrations and the authors were thought to have greatly over-interpreted the results of their studies, the French Ministry of Agriculture and Fish concluded that the study of Richard et al. (2005) provided no information that was of use for human health risk assessment (Committee for Study of Toxicity, 2005). As discussed previously, it is now recognized that testing surfactant like substances in such a test system is not valid.

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