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DEVELOPMENTAL AND REPRODUCTIVE OUTCOMES IN HUMANS AND ANIMALS AFTER GLYPHOSATE EXPOSURE: A CRITICAL ANALYSIS

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Glyphosate is the active ingredient of several widely used herbicide formulations. 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 demonstrated extremely low human exposures as a result of normal application practices. Furthermore, the estimated exposure concentrations in humans are >500-fold less than the oral reference dose for glyphosate of 2 mg/kg/d set by the U.S. Environmental Protection Agency (U.S. EPA 1993). In conclusion, the available literature shows no solid evidence linking glyphosate exposure to adverse developmental or reproductive effects at environmentally realistic exposure concentrations.

Glyphosate, shown in Figure 1, is the active ingredient of several widely used herbicide formulations including Roundup, AquaMaster, and Vision branded products. 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 (the most recent year for which usage statistics are available from the U.S. Environmental Protection Agency [EPA]), an estimated 85–90 million lb of glyphosate was applied in the U.S. agricultural sector alone, making it the number one pesticide active ingredient used in the United States (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 unwanted vegetation, but also in large part due to its relative safety for humans and animals.

As previously noted by Williams et al. (2000), glyphosate has properties of both an acid and a base; consequently, the chemical

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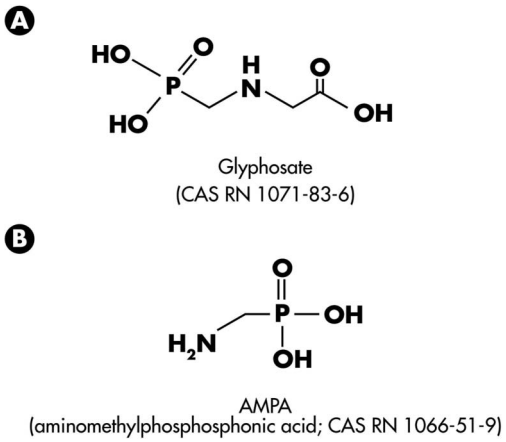

FIGURE 1. Chemical structures of glyphosate (A) and its major breakdown product, AMPA (B).

has several pK_a values (<2, 2.6, 5.6, 10.6; WHO 1994). It has a high water solubility of 12 mg/ml and reported octanol–water partition coefficients (log K_{ow}) of –2.8 and –1.7 (Nielsen et al. 2009; WHO 1994). Glyphosate acid is typically referred to as the technical-grade material, and commercial formulations contain glyphosate in the form of a salt (i.e., potassium, isopropylamine, or ammonium). In addition to glyphosate and water, commercial formulations also typically include a surfactant system, which enables the herbicide product to adhere to the surface of leaves, allowing penetration of the active ingredient (Franz et al. 1997). For instance, in the case of Roundup, there is no single formulation, but rather a range of Roundup-branded products that differ in their surfactants and other ingredients depending on their intended use. One of the more common classes of surfactants used in glyphosate formulations is polyoxyethyleneamines (POEA) (Bradberry et al. 2004). Surfactants may possess their own toxicological properties; these are discussed in more detail in the following and in Bradberry et al. (2004).

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, nonselective 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; Costa 2008). Glyphosate is not anticipated to persist in the environment for extended periods of time following application. 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 nontarget species to glyphosate, thus further increasing its relative safety. Based on these facts, in addition to results from animal toxicity studies, the U.S. Environmental Protection Agency (U.S. 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 (U.S. EPA 1993).

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 U.S. EPA registration requirement for an acute inhalation study was waived for glyphosate due its nonvolatile nature (U.S. EPA 1993).

Data related to dermal exposures indicate extremely low skin absorption rates. In non-Good Laboratory Practices (GLP) studies

conducted with rhesus monkeys, the measured absorption of glyphosate applied to the skin ranged from 0.4 to 2.2% (Wester 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 showed that <2% of Roundup is absorbed as either a concentrated or diluted spray (Franz 1983; Wester et al. 1991). Several recent in vitro human dermal absorption studies have been conducted on glyphosate or glyphosate-based formulations. Nielsen et al. (2009) tested a variety of compounds including glyphosate 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 type of in vitro study is designed to examine the penetration of a substance across excised skin as a measure of potential dermal absorption (OECD 2004). The study by Nielsen et al. (2009) was conservative compared to established guidelines in that (i) the duration of exposure was 48 h without an interim wash; (ii) no tape strips were removed from the skin to differentiate between biologically available glyphosate and that contained in top stratum corneum layers; and (iii) test cells were occluded with parafilm, resulting in hydration of the skin and potentially enhanced dermal permeability. In a later study that involved a similar experimental protocol but with an interim wash at 6 h (Nielsen 2010), glyphosate absorption was only 0.1% of dose and 0.1% was recovered in or on the skin; total glyphosate recovery was 100%. Ward (2009a; 2009b; 2009c) conducted OECD-compliant studies on three different glyphosate-based formulations, testing concentrate and two dilutions representing the range of field concentrations for each of the three formulations (dilutions ranging from 12.5× to 200×). These studies were conservative in that exposures were for a full 24 h with no interim washes. 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 from less than 0.14 to 0.8% for the dilute formulations.

Based on studies conducted in the rat, oral absorption also appears to be limited. Following administration of a single oral dose (5.6–10 mg/kg), approximately 30–36% 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 (Brewster et al. 1991; Chan and Mahler 1992; Williams et al. 2000). 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). Based on its ionic nature and high water solubility, glyphosate does not bioaccumulate to any appreciable levels (Brewster et al. 199; WHO 1994). Following ingestion of a relatively large dose in rats, the blood glyphosate concentrations peaked at around 1–2 h post administration; urinary elimination is complete at approximately 12 h post dosing (Chan and Mahler 1992). Additional data show that glyphosate is poorly metabolized and generally eliminated in the urine and feces unchanged (Brewster et al. 1991).

Despite the well-established safety of glyphosate for humans by regulatory agencies, it was suggested that chronic, low-level exposure may lead to developmental and reproductive health problems, particularly for men and women residing in agricultural areas associated with heavy herbicide use (Solomon et al. 2009). This notion was developed based primarily on results from recently published in vitro and animal studies conducted using glyphosate-based herbicide formulations (Benachour et al. 2007; Dallegrave et al. 2003; 2007; Dariuch et al. 2001; Gasnier et al. 2009; Marc et al. 2005; Pagenelii et al. 2010; Richard et al. 2005; Romano 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 support of regulatory requirements were made available to the authors. These studies were compliant both with contemporary regulatory guidelines and GLP. 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 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 (MOA) could be established to explain how glyphosate may contribute to reproductive and/or developmental problems in humans and other mammals.

Epidemiological Evaluation

Epidemiology is the study of disease patterns and the factors that play a role in their occurrence. The strongest epidemiological designs are cohort and case-control studies. Cohort studies are longitudinal prospective or retrospective investigations of persons exposed to an agent of interest. Exposed and unexposed populations are identified, and the prevalence of the disease or condition of interest within both populations over time is then assessed. Disease occurrences that are followed prospectively are more likely to have accurate exposure information, because confounding factors associated with inaccurate recall are reduced. Retrospective cohort studies rely on the accuracy of information entered in various registries (for example, birth and employment registries) and appropriate exposure recall of interviewees.

Case-control studies identify persons who have developed a particular disease (cases) and then retrospectively investigate the histories and habits of this population to determine whether any differences exist between the case population and another of disease-free individuals (controls) who are matched for age, sex, body weight, and exposure to environmental factors. When evaluating the results of case-control studies, recall bias among the cases and controls, the accuracy of the reporting physician and/or hospital (if birth registries are used), and the possibility of interviewer bias must be considered.

Other types of epidemiological investigations, including cluster analyses, general observational studies, and cross-sectional studies, are less rigorously designed than cohort and case-control studies. Cluster analyses report observations of isolated disease cases, often related to exposures to a specific agent. While considerable detail about each particular case is often available, the small number of cases results in insufficient statistical power to establish an association between exposure and a specific disease. General observational studies investigate whether exposure to an agent is related to the outcome of interest, but the numbers of cases and controls, the selection criteria, and/or the type(s) of controls are not as robust as those in the case-control and cohort studies. Often, general observational studies are based on birth records, employment records, death certificates, or surveys of cases

and controls. Cross-sectional studies are observational studies that primarily ascertain the disease incidence at a moment in time for a given population, as opposed to dealing with individuals and their histories. Thus, cross-sectional studies report disease rates rather than cases, which precludes identifying many confounding factors, which are often based on data for each individual.

Although a substantial body of data exists regarding the adverse reproductive and developmental effects of pesticide exposures in general, 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 11 epidemiology studies evaluating pregnancy outcomes or reproductive health as they relate to glyphosate exposures were identified (Tables 1 and 2). With the exception of Sanin et al. (2009), who only examined glyphosate but did not conduct detailed exposure assessments, none of these studies was designed to specifically assess exposures to glyphosate; rather, these studies address pesticide and/or herbicide exposures, categorized by use or chemical group. In the majority of these publications, glyphosate is only mentioned in passing, or is grouped with other herbicides such as phosphonamino herbicides or organophosphate pesticides (an incorrect classification of glyphosate).

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, 7379 farms in Ontario were identified as likely to be full-time family-run operations. Through telephone screening, a subset of 2946 couples was identified as eligible for study based on residence on or near the farm year-round and the female partner being $\leq$44 years of age. In total, 1898 couples provided completed

TABLE 1. Epidemiological Studies Assessing Glyphosate Exposure and Potential Developmental Effects

Study	Agent	Exposure	Study population	Endpoints	Outcome
Savitz et al. 1997	Glyphosate	Male, self-reported (0–3 mo prior to conception)	3984 pregnancies Ontario Farm Family Health Study (OFFHS)	Miscarriages, preterm deliveries, SGA births	No effect
Arbuckle et al. 2001	Glyphosate	Male or female, self-reported (0–3 mo prior to 1st trimester)	395 spontaneous abortions OFFHS	Spontaneous abortions	No effect
Bell et al. 2001a	Phosphate pesticides	Female, via maternal address (1–20 wk gestation)	74 fetal deaths due to congenital anomalies 20 wk gestation to 24 h after birth	Fetal deaths	Increased odds
Bell et al. 2001b	Phosphate pesticides	Female, via maternal address (1–20 wk gestation)	413 fetal deaths due to causes other than above 20 wk gestation to 24 hr after birth	Fetal deaths	No effect
Rull et al. 2006	Glyphosate	Female, via maternal address (periconception)	731 NTD (anencephaly, spina bifida cystica, other sub-types)	Neural tube defects	No effect
Garry et al. 2002	Herbicides, insecticides, fungicides, fumigants	Male or female, self-reported	1532 live births	Birth defects	Increased risk with exposure to all 4 classes (in column 2)

TABLE 2. Epidemiological Studies Assessing Glyphosate Exposure and Potential Effects of Reproductive Health

Study	Agent	Exposure	Study population	Endpoints	Outcome
Curtis et al. 1999	Glyphosate	Male or female, self reported (0–2 mo prior to pregnancy attempts)	2,012 planned pregnancies OFFHS	Fertility	♀: Decrease ♂: Increase Use on farm: Increase
Larsen et al. 1998	Spermatotoxic. pesticides	Male, self-reported (1 yr prior to child's birth)	904 pregnancies	Time to pregnancy	No effect
Sanin et al. 2009; Solomon et al. 2009	Glyphosate	Female, via residence in region of aerial glyphosate application	2751 first pregnancies	Time to pregnancy	No effect
Greenlee et al. 2003	Herbicides	Female, self-reported (2 yr prior to pregnancy attempts)	322 cases of female infertility	Infertility	Increased
Farr et al. 2004	Glyphosate	Female, self-reported (12 mo previous)	3103 premenopausal women Agricultural Health Study	Menstrual cycle factors (cycle length, intermenstrual bleeding, missed periods)	No effect

questionnaires from the farm operator, husband, and wife (response rate of 64%). These couples reported a total of 5853 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 were 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 5853 OFFHS pregnancies, 3984 were included in this study. Approximately 40% of those included in the analysis occurred 10 yr 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 5 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 backward beyond the 5-yr 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 1 mo or more during the 3 mo 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 5-yr 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, and caffeine use) 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 postconception pesticide exposures on spontaneous abortion, or miscarriage risks. Self-reported miscarriages of less than 20 wk of gestation were divided into 2 groups: those of less than 12 wk of gestation and those occurring between wk 12 and 19 of gestation. Out of a total of 3936 pregnancies, 395 spontaneous abortions were reported; all but 5 were reported to have been medically confirmed and 57% occurred before 12 wk of gestation. The approximate 10% rate for spontaneous abortions reported in this study is substantially lower than the approximate 12–25% rate reported for the general population (Everett 1997; Wilcox et al. 1999). Recall was greater than 5 yr for 64% of the spontaneous abortions and greater than 10 yr 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 postconception period (defined as the three calendar months of the first trimester). Although not specifically discussed in this article, 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 (OR) were calculated. Preconception exposure of fathers to glyphosate showed an elevated risk for spontaneous abortions between wk 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 postconception exposures to glyphosate demonstrated an increased risk of spontaneous abortions either before 12 wk or between wk 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, the results fail to demonstrate a significant association of glyphosate exposure with the risk of spontaneous abortion and need to be considered cautiously in light of substantial exposure misclassification that is likely to have taken place and the lack of adjustment made to the OR to account for the influence of confounding variables.

Fetal deaths. Bell et al. (2001a; 2001b) conducted two case-control studies to examine the possible associations between maternal pesticide exposure and fetal death due to congenital anomalies or other causes. In the congenital anomalies case-control study (Bell et al. 2001a), 73 cases were identified from the 1984 vital statistics records of 10 California counties. Cases were limited to fetal deaths identified as due to congenital anomalies and occurring after 20 wk of gestation; these included 43 neonatal deaths within the first 24 h 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 10 counties 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 1 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 TRS 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 5 separate classes (phosphates, carbamates, pyrethroids, halogenated hydrocarbons, and endocrine disruptors). Glyphosate (referred to as "glyphosphate" in the article) was classified as a phosphate/thiophosphate/phosphonate pesticide (by far, the broadest pesticide category evaluated in the study). Exposures were then analyzed according to 3 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 nonexposure to be no exposure to any of the 5 classes of pesticides during gestational weeks 3–8. Although this method of assigning exposure is not subject to recall bias (a major strength), it still allows for some misclassification. In addition, pregnancies that were exposed only once during a particular exposure period would be analyzed together 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 TRS), no statistically increased OR 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 pyrethroids (discussed in erratum Bell et al. 2001c) and halogenated hydrocarbons. These risks rose as the definition of the exposure period was tapered down from anytime during wk 1–20 to only during wk 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 wk 1–20 of gestation was associated with an OR of 2 (95% CI = 1–4), while exposure during wk 3–8 only was associated with an OR of 3 (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 10 California counties as the previous study. These cases included 86 neonatal deaths within 24 h of birth, but excluded deaths for pregnancies of less than 20 wk, those due to congenital abnormalities, and other causes not likely to be related to environmental exposures (i.e., multiple births and umbilical cord compression). 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 whether 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 were

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% CI 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 3000 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, 1070 volunteered to participate. Enough detailed information regarding reproductive health outcomes and pesticide use was obtained for 695 families (536 with children) and 1532 live births. Births occurred from 1968 to 1998, with over half of the births occurring before 1978 (almost 20 yr 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 6 mo 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 significantly higher than that observed in an earlier cohort study (Garry et al. 1996) that only used medically confirmed cases of birth defects (26.1 vs. 18.9 per 1000 live births). Data suggest 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 quantitatively increased incidence of having children with birth defects compared to use of herbicide alone. 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. Garry et al. (2002) 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). Further, these are parent-reported cases that have not been confirmed through medical records and, as Bloom and Dey (2006) 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 reliable insight concerning the potential adverse 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 (NTD) conducted by the California Birth Defects Monitoring

Program (Shaw et al. 1995; 1999). Cases were confirmed with 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 yr after date of delivery; Shaw et al. 1999) or in-person interviews (on average 5 mo 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 1000-m 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 NTD 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; 2001b), 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 NTD (single pesticide model: OR = 1.5, 95% CI = 1–2.4; 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 1898 couples and 5853 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 2012 pregnancies, 67% of which were conceived at least 5 yr prior to the study and 36% of which occurred at least 10 yr 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 Curtis et al. (1999) indicated that an analysis limited to those pregnancies conceived within 5 years of the study gave results similar to 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 might influence fecundability, including frequency of intercourse, breastfeeding history, reproductive health issues, or menstrual cycle characteristics. 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 2 mo prior. Exposures were also classified by whether the husband, wife, or both participated in a pesticide activity. For each exposure type, Curtis et al. (1999) 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 3 mo 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 = 0.3–1.26). The finding may also 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. In contrast to the preceding finding, glyphosate use on the farm (with no reported pesticide-related activities by women or men) was associated with a numerical increase, albeit not statistically significant, in fecundability (CFR = 1.26, 95% CI = 0.94–1.69). Further, men's exposure to glyphosate demonstrated a statistically significant rise in fecundability (CFR = 1.3, 95% CI = 1.07–1.56); however, this association was downplayed 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 in Denmark. In total, 904 male farmers ranging in age from 18 to 50 yr 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 Larsen et al. (1998), 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 from the study report, those showing time to pregnancy greater than 12 mo 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 = 0.75–1.4). 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 = 1.07–2.4). In addition, the use of three or more "spermatotoxic" pesticides in the year prior to the youngest child's birth exerted 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 5 yr for 52% of the traditional farmers who sprayed pesticides, these data likely suffer from some exposure misclassifications. Further, because those without children and those with greater than 12 mo to pregnancy were excluded from the analyses, it is possible that an effect of

pesticide exposure may have been underestimated.

Time to pregnancy was also evaluated in women from five Colombian regions that differed in glyphosate use for the eradication of illicit crops (Sanin et al. 2009; Solomon et al. 2009). The regions for study included Boyacá and Sierra Nevada de Santa Marta, both of which did not conduct aerial spraying of glyphosate; Nariño and Putamayo, both of which conducted aerial spraying of glyphosate for the control of illicit crops; and Valle del Cauca, a more developed region that reported aerial spraying of glyphosate on sugar cane crops. In total, 2751 women aged 25 yr of older who reported their first pregnancy within the last 5 yr and no contraceptive use in the year prior to pregnancy were enrolled in the study between August 2004 and February 2005. Enrollments were conducted through house-to-house visits. Refusals to participate were only reported in Valle del Cauca and amounted to approximately 3% of the women queried in that region. Total participants from each region varied between 531 and 582. Information on the number of months of sexual intercourse prior to pregnancy was collected. Data on potential confounders were also collected, including age at first pregnancy, partner relationship, work and medical histories, lifestyle practices (smoking and use of drugs, coffee, and alcohol), and the work status and lifestyle practices of the father. Specific information regarding glyphosate exposures was not gathered; rather, exposures were assumed to relate to the degree of glyphosate aerial application registered for the region. Fecundability OR were calculated with 95% CI. In a restricted analysis, those women who reported seeking medical treatment for possible fertility issues ($n = 159$) were excluded. Substantial differences in the mean time to pregnancy in months were observed among the 5 Colombian regions of interest, with the lowest mean of only 3 mo reported in Boyacá and the greatest mean of 14 mo reported in Valle del Cauca. These differences in time to pregnancy did not correspond, however, with the use of glyphosate for aerial eradication of illicit crops. Thus, these data do not indicate an association between glyphosate use and increases in time to pregnancy.

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 yr, who sought treatment at an obstetrics/gynecology (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 mo 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 yr, 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 mo 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 2 yr 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, to 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 min, respectively), indicating possible recall bias. An analysis of agricultural variables showed that a woman's exposure to herbicides at any time during the 2-yr period prior to trying to conceive was statistically associated with an elevated 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 OR 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 2-yr 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.

Menstrual cycle characteristics. The Agricultural Health Study is a prospective cohort study with more than 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 in phase I of the study. Based on these data, Farr et al. (2004) examined the effects of pesticide use on the menstrual cycle characteristics of 3103 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 and were identified as premenopausal and between the ages of 21 and 40 yr, 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 d or less), long cycles (36 d or more), irregular cycles, missed periods (no periods for an interval of more than 6 wk in the last 12 mo), and bleeding/spotting between periods within the last 12 mo. 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 d, 1–9 d, and 10 d or more. 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. 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). Controlling for the average number of days worked in the fields quantitatively buffered these findings (OR 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 did not address potential effects of glyphosate exposure on 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 11 such studies were identified, and none of these—with the exception of Sanin et al. (2002)—was designed to specifically assess the effects of glyphosate exposure. 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 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, glyphosate was inappropriately included 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 (Arbuckle et al. 2001; Bell et al. 2001b; Rull et al. 2006; Savitz et al. 1997). Of five studies that assessed the potential reproductive health effects of glyphosate (Curtis et al. 1999; Farr et al. 2004; Greenlee et al. 2003; Larsen et al. 1998; Sanin et al. 2009), none reported significant adverse outcomes associated with exposure. Greenlee et al. (2003) suggested that female infertility may be influenced by herbicide exposure, but 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 significant 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 U.S. EPA or Organization for Economic Cooperation and Development (OECD); others used limited numbers of animals per group, inadequate study designs, inappropriate controls, and inadequately-identified test materials. 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 glyphosate 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, 1000, or 3500 mg/kg/d glyphosate on gestational days (GD) 6–19 (25 animals per group). The high dose in this study is 1750-fold higher than the oral reference dose of 2 mg/kg/d set for glyphosate by the U.S. EPA (1993) and more than 10,000-fold higher than the highest estimated dose of glyphosate measured in biomonitoring studies (discussed in a subsequent section of this article). 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 developmental effects of treatment assessed. The numbers of corpora lutea, implantations, resorptions, and 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 were prepared for evaluation of skeletal morphology. Gross malformations were not reported separately from soft tissue and skeletal malformations.

Clinical signs including soft stools, diarrhea, breathing rattles, and inactivity were noted in rats dosed with 3500 mg/kg/d 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

TABLE 3. Developmental Animal Studies Conducted using Gavage Administration of Glyphosate, POEA, AMPA, or Roundup

Animal model (number per group)	Agent (exposure duration)	Dose (mg/kg/d)	Number of gravid females	Number (mean) of corpora lutea	Number (mean) of implantations	Number (%) resorptions	Number (%) live fetuses	Mean fetal weight (g)	Number of fetuses (litters) with malformations	Number (%) fetuses with malformations			Maternal effects	Reference
										Gross	Visceral	Skeletal		
Charles River COBS CD rats (25)	Glyphosate (GD 6–19)	0	22	349 (15.9)	330 (15.0)	14	316	3.5	3 (3)	nr	2 (2)	1 (1)		IRDC 1980a
		300	20	303 (15.2)	241 (12.1)**	4	237*	3.7	0 (0)	nr	0 (0)	0 (0)		
		1000	21	338 (16.1)	310 (14.8)	10	300	3.6	0 (0)	nr	0 (0)	0 (0)		
		3500	23	237 (14.8)	217 (12.8)*	21*	196*	3.2**	10 (3)	nr	7 (2)	9 (2)	6/25 deaths; various signs of clinical toxicity; decreased weight gain due to weight loss on GD 6–9	
Dutch belted rabbits (16)	Glyphosate (GD 6–27)	0	14	108 (9.0)	71 (5.9)	8	63	33.4	0 (0)	nr	0 (0)	0 (0)	2/16 dams aborted on GD 22; soft stools/diarrhea	IRDC 1980b
		75	16	152 (10.1)	120 (8.0)	6	**114***	30.9	3 (3)	nr	0 (0)	3 (3)	1/16 deaths on GD 26; soft stools/diarrhea	
		175	14	116 (10.5)	67 (6.1)	2	65	29.9	2 (2)	nr	0 (0)	2 (2)	1/16 dams aborted on GD 27; 2/16 deaths on GD 22 and 25; increased soft stools/diarrhea	
		350	16	51 (8.5)	43 (7.2)	5	38	29.3	2 (1)	nr	2 (1)	0 (0)	1/17 dams aborted on GD 23; 10/17 deaths by GD21; increased soft stools/diarrhea, nasal discharge	
SD Crl:CD BR rats (25)	MON 0818 (POEA) (GD 6-15)	0	24	376	341	16 (6.0)	325 (94.0)	3.5	2 (2)	1 (0.3)	1 (0.3)	0 (0)		Holson 1990
		15	23	377	316	21 (6.7)	295 (93.3)	3.6	2 (2)	2 (0.7)	0 (0)	0 (0)		
		100	22	356	320	16 (5.3)	304 (94.7)	3.6	0 (0)	0 (0)	0 (0)	0 (0)	Infrequent clinical signs; decreased food consumption	
		300	15	344	216	11 (5.1)	205 (94.9)	3.4	4 (4)	1 (0.5)	3 (1.5)	1 (0.5)	6/25 deaths; clinical signs; decreased weight gain; decreased food consumption	

(*Continued*)

TABLE 3. (*Continued*)

Animal model (number per group)	Agent (exposure duration)	Dose (mg/kg/d)	Number of gravid females	Number (mean) of corpora lutea	Number (mean) of implantations	Number (%) resorptions	Number (%) live fetuses	Mean fetal weight (g)	Number of fetuses (litters) with malformations	Number (%) fetuses with malformations			Maternal effects	Reference
										Gross	Visceral	Skeletal		
SD Crl:CD BR rats (25)	AMPA (GD 6-15)	0	24	387	361	16 (4.4)	345 (95.6)	3.5	2 (2)	1 (0.3)	1 (0.3)	0 (0)		Holson 1991
		150	24	407	366	19 (5.3)	347 (94.7)	3.5	4 (4)	1 (0.3)	2 (0.6)	3 (0.9)		
		400	24	394	360	14 (3.9)	346 (96.1)	3.4	0 (0)	0 (0)	0 (0)	0 (0)	Increased mucoid feces/soft stools	
		1000	24	397	363	14 (3.8)	349 (96.2)	**3.3***	2 (2)	2 (0.6)	0 (0)	0 (0)	Increased mucoid feces/soft stools	
Wistar rats (14–16)	Roundup (GD 6–15)	0	15	171^(11.4)	157^(10.5)	3^(2.4)	154^	5.1	nr	0 (0)	nr	24^@(15.4)		Dallegrave et al. 2003
		500	15	174^(11.6)	147^(9.8)	#^(3.3)	148^	5	nr	3^(2.0)	nr	49^(33.1)		
		750	16	162^(10.1)	186^(11.6)	24^(2.6)	162^	5.1	nr	1^(0.6)	nr	68^(42.0)	Decreased food consumption	
		1000	14	90^(12.9)	80^(11.4)	5^(3.8)	75^	5.1	nr	0 (0)	nr	43^(57.3)	7/14 dams died GD 7–14; decreased weight gain; decreased food consumption	

Note. *Statistically different from control, $p < .05$; **statistically different from control, $p < .01$. ^, Authors did not distinguish between live and dead fetuses; means reported in paper for high-dose group based on only seven surviving dams; number of corpora lutea and implantations have been calculated based on the means and number of dams reported in the paper; number of fetuses with gross and skeletal malformations have been calculated based on the percentages and number of fetuses reported in the paper; number of resorptions have been calculated as the difference between the number of fetuses reported and the calculated number of implantations. #, Number of resorptions cannot be calculated from the provided data. @, Number of animals with skeletal malformations cannot be calculated from the percentage provided by the authors.

six rats at necropsy. Maternal body weights in the glyphosate-treated groups were not significantly different from control, although the body weight gain in the high-dose group was numerically decreased compared to control. Statistically significant decreases in the mean numbers of implantations and viable fetuses per dam in the 300-mg/kg/d treatment group were noted; however, because no marked effects of treatment were noted in the next higher dose group of 1000 mg/kg/d (and implantations occurred before treatment), these changes were attributed to random chance. In the 3500-mg/kg/d 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 (possibly related to reduced maternal weight gains at the high dose). No significant change in the mean number of corpora lutea per dam was 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 apparent malformations were observed in fetuses from the 300- and 1000-mg/kg/d 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 3500-mg/kg/d glyphosate group, 10 fetuses had malformations; these included 7 soft-tissue malformations and 9 skeletal malformations. Upon closer examination, however, it was 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). Based on the types of malformations and their occurrence in limited litters, IRDC (1980a) concluded that these findings likely represented a litter effect of genetic origin. An increased incidence of unossified sternebrae (a variation, possibly related to the reduced fetal weights and a developmental delay) was also reported in fetuses at 3500 mg/kg/d. Overall, this study indicates a no-observable-adverse-effect level (NOAEL) of 1000 mg/kg/d glyphosate for both maternal and developmental toxicity.

In IRDC (1980b), female Dutch belted rabbits were inseminated on GD 0 using semen from 4 proven male rabbits. Impregnated does were administered 0, 75, 175, or 350 mg/kg/d 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, and 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 rise in incidence in dams treated with 175 and 350 mg/kg/d glyphosate compared to controls. Animals at 350 mg/kg/d 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 2 rabbits from the control group, and in 1 rabbit in each of the 175- and 350-mg/kg/d 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, 2, and 10 rabbits died before the end of study in the 75-, 175-, and 350-mg/kg/d glyphosate treatment groups, respectively. The causes of maternal death were determined for 5 of 13 animals, but were not consistent across the group. A statistically significant elevation in number of viable fetuses per dam treated with 75 mg/kg/d was noted, but this result was considered to be a random occurrence because it was not

observed in the 2 higher treatment groups. Compared to controls, glyphosate treatment exerted no marked effect on the numbers of corpora lutea, implantations, resorptions, live and dead fetuses, fetal sex ratios, or fetal weights. Glyphosate treatment also had no significant effect on the incidence of fetal malformations and variations. Based on mortality and clinical signs at 350 mg/kg/d, the NOAEL for maternal toxicity is considered to be 175 mg/kg/d. Although no apparent developmental toxicity was observed at any dose, 175 mg/kg/d is considered the NOAEL for developmental toxicity as well because too few fetuses were available at the high dose of 350 mg/kg/d for adequate toxicological assessment.

POEA—Surfactants used in a number of commercial herbicide formulations. One GLP- and guideline-compliant developmental toxicity study was conducted with 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/d 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 visceral dissection and alizarin staining for osseous effects. In this study, 6/25 animals treated with 300 mg/kg/d POEA died before the end of study and clinical signs of maternal toxicity were observed in the remaining animals at this dose level. In addition, significant decreases in weight gain and food consumption during the treatment period were observed. Infrequent signs of clinical toxicity and significantly lower food consumption on GD 6–9 were recorded for animals in the 100-mg/kg/d treatment group. However, despite these obvious signs of maternal toxicity, no significant compound-related developmental effects on the offspring were observed. The rate of malformations in the treated groups was within the historical control range for the lab. From these results, Holson (1990) concluded that 15 mg/kg/d POEA was the NOAEL for maternal toxicity and 300 mg/kg/d POEA was the NOAEL for developmental toxicity.

Aminomethylphosphonic acid (AMPA)—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/d 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 were 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 1000 mg/kg/d AMPA and these effects appeared to rise in a treatment-related manner. Offspring were examined by visceral dissection and alizarin staining for osseous effects. No marked developmental effects of treatment on the offspring were observed except for a statistically significant decrease in fetal weights at 1000 mg/kg/d. This finding was within the range of historical control data from the laboratory at which the study was conducted and was influenced by the results of two litters. All malformations in the treated groups were considered to be of spontaneous origin and unrelated to treatment. Based on these results, Holson (1991) concluded that 400 mg/kg/d AMPA was the NOAEL for both maternal and developmental toxicity.

Commercial herbicide formulation. One 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 4 treatment groups and dosed once daily by gavage with 0, 500, 750, or 1000 mg/kg/d "glyphosate-Roundup" on GD 6–15. The treatment description provided was ambiguous as to whether the dosages stated were

for "Roundup" or the active ingredient, glyphosate. Body weights were recorded daily, and food and water consumption was recorded at appropriate intervals. On GD 21, animals were sacrificed and the effects of treatment on fetal development 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 half due to animal deaths. Furthermore, few data are actually presented in the article. 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 article to what values these reported intakes are normalized. Similarly, fetal findings are presented as percentages or unsubstantiated mean values throughout the article, which complicates interpretation. When one converts these mean reproductive indices to actual totals, one discovers that 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/d treatment group, more fetuses were reported than implantation sites. This brings into question the reported resorption rate for this treatment group. Further, in the 750-mg/kg/d treatment group, more implantation sites than corpora lutea were reported.

Dallegrave et al. (2003) reported a dose-related increased incidence of skeletal alterations of 15.4, 33.1, 42, and 57.3% in control, 500-, 750-, and 1000-mg/kg/d treatment groups, respectively. The most frequently observed alterations included incomplete ossification of the skull and enlarged fontanels. Interestingly, examination of the list of skeletal alterations observed in this study showed 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 could 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 data, it is not possible to draw any conclusions regarding the developmental effects of "Roundup" treatment from this article. Furthermore, because a commercial formulation was used, it is not possible to attribute any observed effects to glyphosate specifically.

Reproductive Studies

Glyphosate. Three multigenerational reproductive studies tested glyphosate (Table 4), two in Sprague-Dawley rats (Schroeder 1981; 1982; Reyna 1990) and one in Wistar-derived rats (Moxon 2000). Although conducted prior to the establishment of GLP, the Schroeder (1981; 1982) 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/d throughout. The test substance was administered starting 63 d 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 d between weaning and mating, resulting in 2 sets of litters per generation. The first litter of each generation was sacrificed at weaning and

TABLE 4. Reproductive Animal Studies Conducted Using Glyphosate or POEA Administration in the Diet

Animal model (number/group)	Agent (exposure duration)	Mating (resulting progeny)	Dose	Number (%) gravid females	Mean gestational length (days)	Number dead fetuses (mean)	Number live fetuses (mean)	Percent PND0 male pups	Mean number pups weaned/ litter	Mean offspring weight (g)			Number surviving offspring (%)		Reference
										PND0	PND4	PND21	PND0–4	PND4–21	
SD Crl: CD rats^ (24 F; 12 M)	Glyphosate (63 d prior to mating through weaning of litter F3b)	F0 (F1a)	0 mg/kg/d	19 (95)	22.1	2 (0.1)	218 (11.5)	50.3	10.7	6	9.9	41.1	210 (96.3)	192 (98.5)	Schoeder 1981; 1982
			3 mg/kg/d	21 (95.5)	21.8	3 (0.1)	268 (12.8)	48.5	12.4	5.8	9.3	37.7	251 (93.7)	247 (98.4)	
			10 mg/kg/d	16 (84.2)	21.8	4 (0.3)	196 (12.3)	51.5	11.9	5.9	9.4	39.7	194 (99.0)	192 (99.0)	
			30 mg/kg/d	19 (90.5)	21.8	1 (0.1)	221 (11.6)	50.7	11.3	6	9.6	39.2	217 (98.2)	215 (99.1)	
		F0 (F1b)	0 mg/kg/d	19 (95)	22	3 (0.2)	223 (11.7)	47.1	11.3	6.1	9.9	40.9	218 (97.8)	215 (98.6)	
			3 mg/kg/d	19 (82.6)	21.8	11 (0.6)	232 (12.2)	50	11.4	6.1	9.7	43.2	223 (96.1)	206 (92.4)**	
			10 mg/kg/d	12 (70.6)	22	4 (0.3)	153 (12.8)	47.1	10.9	5.8	9	37.9	145 (94.8)	120 (82.8)**	
			30 mg/kg/d	18 (81.8)	21.9	5 (0.3)	226 (12.6)	50	11.4	6.2	9.9	36.9	225 (99.6)	194 (90.7)**	
		F1 (F2a)	0 mg/kg/d	18 (100)	21.9	3 (0.2)	216 (12.0)	43	11.7	5.8	9.4	41	201 (93.1)	199 (99.0)	
			3 mg/kg/d	20 (87)	21.8	0 (0.0)	236 (11.8)	55.9	11.6	6	9.7	43.4	231 (97.9)*	231 (100)	
			10 mg/kg/d	17 (94.4)	21.9	0 (0.0)	216 (12.7)	48.6	12.4	6	9.1	39.7	214 (99.1)**	211 (98.6)	
			30 mg/kg/d	18 (94.7)	22	7 (0.4)	207 (11.5)	49.1	11.1	6.2	9.4	40.3	206 (99.5)**	200 (97.1)	
		F1 (F2b)	0 mg/kg/d	15 (88.2)	21.9	6 (0.4)	186 (12.4)	48.4	11.9	5.9	9.4	41.1	178 (95.7)	178 (100)	
			3 mg/kg/d	15 (78.9)	21.9	6 (0.4)	187 (12.5)	48.7	12.7	5.7	9.2	41.1	166 (88.8)*	165 (99.4)	
			10 mg/kg/d	14 (82.4)	22.1	3 (0.2)	184 (13.1)	49.5	12.7	5.8	9.5	41.3	181 (98.4)	178 (98.3)	
			30 mg/kg/d	14 (73.7)	22.1	4 (0.3)	147 (11.3)	51	11.1	6.4	10.3	41.3	144 (98.0)	144 (100)	
		F2 (F3a)	0 mg/kg/d	23 (95.8)	21.9	5 (0.2)	268 (11.7)	51.4	11	6	9.5	37.1	266 (99.3)	254 (95.5)	
			3 mg/kg/d	20 (100)	22	18 (0.9)	222 (11.1)	49.8	10.6	6.1	9.4	36.8	219 (98.6)	190 (86.8)*	
			10 mg/kg/d	16 (80)	21.9	2 (0.1)	202 (12.6)	48.7	11.8	6	9.5	37.3	202 (100)	188 (93.1)	
			30 mg/kg/d	17 (94.4)	21.9	5 (0.3)	200 (11.8)	54.4	11	6.3	9.8	36.7	198 (99.0)	187 (94.4)	
		F2 (F3b)	0 mg/kg/d	22 (95.7)	21.9	5 (0.2)	247 (11.2)	45.7	11.3	5.9	8.8	38.1	241 (97.6)	237 (98.3)	
			3 mg/kg/d	16 (84.2)	21.9	6 (0.4)	197 (12.3)	46.2	12.7	5.8	9	39.6	192 (97.5)	191 (99.5)	
			10 mg/kg/d	16 (80)	22.1	2 (0.1)	208 (13.0)	45.7	12.4	6.1	9.4	39.9	202 (97.1)	198 (98.0)	
			30 mg/kg/d	19 (90.5)	21.9	10 (0.5)	187 (9.8)	45.5	9.9	6.3	9.1	38.5	183 (97.9)	178 (97.3)	
Sprague Dawley Rats#° (30 F; 30 M)	Glyphosate (11 wk premating through weaning of litter F2b)	F0 (F1)	0 ppm	24 (82.8)	22.29	1 (0.0)	318 (13.3)	49.7	na	6.1	9.4#	52	307 (95.4)	(99.0)	Reyna 1990
			2000 ppm	29 (96.7)	22.22	5 (0.2)	362 (12.5)	53.5	na	6.1	9.3#	50.4	352 (97.5)	(99.6)	
			10,000 ppm	28 (96.6)	22.5	3 (0.1)	355 (12.7)	51	na	6.3	9.5#	49.8	352 (99.4)*	(99.6)	
			30,000 ppm	28 (93.3)	22.26	4 (0.1)	323 (11.5)	50.5	na	6.3	9.6#	46.9**	323 (100)**	(98.4)	
		F1 (F2a)	0 ppm	28 (93.3)	22.44	2 (0.1)	337 (12.0)	45.1	na	6.1	9.7	53.3	325 (96.7)	(100.0)	
			2000 ppm	24 (85.7)	22.58	6 (0.3)	308 (12.3)	52.3	na	6	9.4	51.2	284 (90.9)	(96.7)	
			10,000 ppm	24 (82.8)	22.61	4 (0.2)	275 (11.5)	48	na	6.1	9.5	50.1*	263 (96.2)	(99.5)	
			30,000 ppm	26 (89.7)	22.58	0 (0.0)	281 (10.8)	49.1	na	6.3	9.8	45.9**	279 (99.4)	(100.0)	
		F1 (F2b)	0 ppm	16 (64.0)	22.44	2 (0.1)	191 (11.9)	49.7	na	6.2	9.8	50.8	188 (98.8)	(100.0)	
			2000 ppm	21 (84.0)	22.55	4 (0.2)	217 (10.9)	55.8	na	6	9.7	51.8	193 (91.1)	(99.3)	
			10,000 ppm	19 (79.2)	22.37	3 (0.2)	250 (13.2)	54.4	na	6.2	9.3	51	239 (96.5)	(94.1)	
			30,000 ppm	25 (96.2)	22.5	4 (0.2)	268 (10.7)	48.1	na	6.3	9.7	43.4**	258 (96.9)	(100.0)	

Alpk:AP$_f$SD (Wistar-derived) rats† (26 F; 26 M)	Glyphosate (10 wk premating through weaning f litter F2a)	F0 (F1a)	0 ppm	23 (88.5)	22.3	18	277 (12.1)	53.8	10.6	5.6	9.1	42.7	224 (89.3)	223	Moxon 2000
			1000 ppm	22 (84.6)	22.3	8	253 (11.5)	49.8	10.0	6.0	8.8	41.4	225 (88.4)	219	
			3000 ppm	22 (84.6)	22.2	3	261 (11.8)**	53.3	10.9	5.8	8.7	40.4	228 (92.7)	228	
			10,000 ppm	24 (92.3)	22.2	10	291 (12.0)	50.5	11.2	5.9	8.3	38.5*	262 (94.8)**	257	
		F1 (F2a)	0 ppm	22 (84.6)	22.3	13	226 (10.2)	50.9	9.8	6.2	9.5	43.6	207 (96.2)	206	
			1000 ppm	23 (88.5)	22.2	9	254 (10.9)	52.2	9.9	6.1	9.8	45.5	218 (90.2)*	218	
			3000 ppm	21 (80.8)	22.1	3	251 (13.0)**	53.8	11.9*	6.1	9.2	42.5	227 (93.0)*	226	
			10,000 ppm	25 (96.2)	**22.0****	8	296 (11.9)	54.4	11.1	6.0	9.3	43.9	278 (93.5)	278	
Sprague-Dawley rats¶(20 F; 20 M for F0; 31/32 for controls, 24/26 for 1000 ppm)	MON 0818 (POEA)(10 wk premating through PND4 of litter F2a)	F0 (F1a)	0 ppm	16 (80.0)	22.3	nr	NR (13.9)	nr	nr	7.3	9.9	54.2	NR (97.9)	NR (98.4)	Knapp 2007
			100 ppm	15 (78.9)	22.3	nr	NR (13.1)	nr	nr	7.4	10.5	55.4	NR (93.8)	NR (98.3)	
			300 ppm	14 (73.7)	22.2	nr	NR (13.9)	nr	nr	7.2	10.0	54.6	NR (97.7)	NR (100)	
			1000 ppm	15 (88.2)	22.2	nr	NR (11.8)	nr	nr	7.3	9.8	54.8	NR (82.2)	NR (98.1)	
		F1 (F2a)	0 ppm	28 (93.3)	21.9	nr	NR (14.4)	nr	nr	7.0	9.8	N/A	nr	N/A	
			100 ppm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
			300 ppm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	
			1000 ppm	21 (95.5)	21.9	nr	NR (14.8)	nr	nr	7.2	9.8	N/A	nr	N/A	
Sprague Dawley rats+ (12 F; 12 M)	MON 8109 (POEA)		0 ppm	11 (91.7)	21.5	nr	NR (14.4)	nr	nr	6.6	8.5	N/A	NR (94.2)	N/A	Knapp 2008
			30 ppm	11 (91.7)	21.9	nr	NR (13.9)	nr	nr	6.9	9.1	N/A	NR (97.0)	N/A	
			100 ppm	10 (83.3)	21.9	nr	NR (13.9)	nr	nr	6.7	8.7	N/A	NR (88.7)	N/A	
			300 ppm	12 (100)	21.8	nr	NR (14.8)	nr	nr	6.9	9.3	N/A	NR (95.9)	N/A	
			2000 ppm	12 (100)	21.9	nr	NR (8.0)**	nr	nr	6.2	8.6	N/A	NR (36.3)**	N/A	
	MON 0818(POEA)		1000 ppm	12 (100)	21.7	nr	NR (13.6)	nr	nr	6.7	8.7	N/A	NR (98.7)	N/A	

Note. *Statistically different from control, $p < .05$; **statistically different from control, $p < .01$. ^, PND4-21 survival data for F1a control group excludes litter of single female which gained a pup during this period; PND4–21 survival data for F1b high dose group exclude litter of single female that escaped from cage on PND20. #, Average glyphosate intake in the 2000-, 10,000-, and 30,000-ppm groups during the premating period reported to be 132, 666, and 1983 mg/kg/d, respectively, for F_0 males; 160, 777, and 2322 mg/kg/d, respectively, for F_0 females; 140, 711, and 2230 mg/kg/d, respectively, for F_1 males; and 163, 804, and 2536 mg/kg/d, respectively for F_1 females; PND4 fetal weights reported preculling of litters. †, Average glyphosate intake in the 1000-, 3000-, and 10,000-ppm groups during the premating period reported to be 99, 293, and 985 mg/kg/d, respectively, for F_0 males; 104, 323, and 1054 mg/kg/d, respectively, for F_0 females; 117, 352, and 1161 mg/kg/d, respectively, for F_1 males; and 123, 371, and 1218 mg/kg/d, respectively, for F_1 females; day of birth designated PND1 in study; for purposes of data presentation in table, day of birth is redesignated as PND0; pups weaned on PND29 (PND28); mean offspring weights in table average of mean weights for males and females separately; PND4–21 percent survival not calculated. ¶, Average POEA intake in the 100-, 300-, and 1000-ppm groups during the premating period reported to be 6, 18 and 60.5 mg/kg/d, respectively, for F_0 males and 7, 20.7, and 70.4 mg/kg/d, respectively, for F_0 females; mean offspring weight presented in table is the average of mean male and female pup weights combined; for the F_1 generation, only the control and high-dose groups were mated and pups were sacrificed on PND4; NR = not reported; N/A = not applicable. +, Average MON 8109 intake in the 3-0, 100-, 300-, and 2000-ppm groups during the premating period reported to be 2, 8, 23, and 134 mg/kg/d, respectively, in males and 3, 9, 26, and 148 mg/kg/d, respectively, in females; average MON 0818 intake reported to be 76 and 86 mg/kg/d in males and females, repspectively, in the 1000-ppm group; exposures were for 14 d premating through lactation; pups sacrificed on PND4; mean offspring weight presented in table is the average of mean male and female pup weights combined; NR = not reported; N/A = not applicable.

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 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. In addition, 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 (Schroeder 1982); further, it was not observed in a second study (Reyna 1990; see later discussion) 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 conducted according to established U.S. EPA guidelines of the time. In this study, male and female Sprague-Dawley rats (30/gender/group) were fed diets containing 0, 2000, 10,000, or 30,000 ppm glyphosate starting approximately 11 wk prior to mating. It is of note that the high dose exceeds by 50% the current limit dose for dietary studies (20,000 ppm). Also, the lowest dietary concentration (2,000 ppm) is almost sevenfold higher than the highest dietary concentration (300 ppm) used in the study of Schroeder (1981; 1982). These diets continued to the end of study and throughout all generations. Glyphosate intakes during the premating period averaged 132, 666, and 1983 mg/kg/d for F_0 males and 160, 777, and 2322 mg/kg/d for F_0 females in the 2000-, 10,000-, and 30,000-ppm groups, respectively; average compound intakes during the premating period were slightly higher for the F_1 generation. Litters of the first generation were culled to 8 pups each on PND 4 and weaned on PND 21, at which time 30 rats/gender/group were randomly selected for creation of the F_2 generation. The selected animals were allowed a 14-wk 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 8 pups each on PND 4. F_0 and F_1 adults were examined by gross necropsy. All culled pups, those that died postnatally, those not selected for mating, and all F_{2a} and F_{2b} weanlings were also 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 weanling per gender per litter from these treatment groups of the F_{2b} generation.

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 exerted no marked 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 numerically 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 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. It was postulated that as pups began supplementing their milk intake with consumption of the prepared diets toward 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 694 mg/kg/d, per Williams et al., 2000) is considered the NOAEL for systemic toxicity and 30,000 ppm (approximately 2132 mg/kg/d, per Williams et al., 2000) is considered the NOAEL for reproductive and developmental toxicity.

Moxon (2000) conducted an investigation according to more current U.S. EPA guidelines. In this study, male and female Wistar-derived rats (26/gender/group) were fed diets containing 0, 1000, 3000 and 10,000 ppm glyphosate starting 10 wk before mating. These diets continued to the end of study and throughout two generations. Glyphosate intakes during the premating period averaged 99, 293, and 985 mg/kg/d for F_0 males and 104, 323, and 1054 mg/kg/d for F_0 females in the 1000-, 3000-, and 10,000-ppm groups, respectively; average compound intakes during the premating period were slightly higher for the F_1 generation. The day of birth was designated PND1. The study did not report whether litters were culled. F_{1A} litters were weaned on PND 29 and 26 rats/gender/group selected to become the F_1 parental generation. Mating for production of the F_{2A} litter commenced after another 10-wk premating period. Males were terminated following littering; females were terminated on or soon after PND 29, the day of weaning.

Treatment-related reduced body weights were noted for F_1 males at 10,000 ppm. However, glyphosate did not adversely affect reproductive performance and exerted no adverse effect on pup survival, litter size, or the pup sex ratio for either the F_{1A} or F_{2A} litter. Although pup birth weight was not affected by treatment in both the F_{1A} and F_{2A} litters, F_{1A} pup body weights in the high-dose group

were lower than for controls throughout the lactation period; this finding was not observed for the F_{2A} litters. F_1 males selected for mating had a subsequent reduction in body weight during the premating period. Preputial separation and day of vaginal opening were both unaffected by glyphosate treatment in the F_1 animals (Table 5). Further, no marked effects of treatment were noted on sperm (sperm number, motility or morphology; see Table 5) in F_0 and F_1 males or on the number of primordial and small growing ovarian follicles in high-dose F_1 females (F_0 animals and other dose groups were not evaluated; data not shown). Estrous cycle length was significantly reduced at the high dose in F_1 females; however, because the change was marginal, it was not considered to be treatment related. Glyphosate treatment did not significantly affect F_0 or F_1 organ weights and was not associated with any macroscopic or microscopic findings in either parental animals or pups. Due to the reduced body weights at the high dose of F_{1A} pups during lactation and F_1 males during the premating period, the systemic and offspring/developmental NOAEL were both 3000 ppm (approximately 335 mg/kg/d during the premating period). Due to the absence of effects on fertility and reproductive performance, the reproductive NOAEL is considered to be the highest dose tested, 10,000 ppm (approximately 1105 mg/kg/d during the premating period).

POEA. Two reproductive/developmental screening studies were conducted with POEA surfactants covering a range of carbon chain lengths and degrees of polyalkoxylation (Tables 4 and 5; Knapp 2007; 2008). In the first study (Knapp 2007), groups of male and female Sprague-Dawley rats (20/gender/group) were administered a POEA incorporated into the diet at concentrations of 0, 100, 300, and 1000 ppm for 70 d premating through mating (males) or through mating, gestation, and lactation (females). POEA intake during the premating period averaged 6, 18, and 60.5 mg/kg/d for F_0 males and 7, 20.7, and 70.4 mg/kg/d for F_0 females in the 100-, 300-, and 1000-ppm groups, respectively. F_1 pups

TABLE 5. Sexual Maturation, Sperm Parameters, and Estrous Cyclicity Data from Glyphosate and POEA Reproductive Studies in Rats

Animal model (number per group)	Agent	Generation	Dose (ppm)	Anogenital distance (mm), M/F	Day of preputial separation	Day of vaginal opening	Right cauda weight (g)	Normal sperm (%)	Sperm motility (%)	Straight line velocity (μm/s)	Number of sperm (10^7/g cauda)	Number of sperm (10^7/g testis)	Estrous cycle length (d)	Reference
Alpk:AP$_i$SD (Wistar-derived) rats# (26 F; 26 M)	Glyphosate	F0	0	NA	NA	NA	0.258 ± 0.031	97.9	85.9 ± 8.8	55.7 ± 7.9	513 ± 160	55 ± 7	3.97 ± 0.87	Moxon 2000
			1000	NA	NA	NA	**0.238 ± 0.025***	98.1	81.5 ± 11.5	55.4 ± 11.3	469 ± 213	NA	4.38 ± 1.25	
			3000	NA	NA	NA	0.247 ± 0.033	95.9	83.5 ± 16.1	56.4 ± 10.8	477 ± 182	NA	3.88 ± 0.68	
			10,000	NA	NA	NA	0.256 ± 0.024	97.9	85 ± 8.5	56.3 ± 10.3	550 ± 310	53 ± 10	3.67 ± 0.68	
		F1	0	NA	47.1 ± 2.3	35.3 ± 1.3	0.255 ± 0.034	99.0	78.1 ± 15.9	48.9 ± 12.4	444 ± 183	56 ± 7	4.26 ± 0.58	
			1000	NA	46.6 ± 1.7	35.3 ± 1.5	0.263 ± 0.037	99.0	82.7 ± 18.8	48.1 ± 15.7	503 ± 308	NA	4.12 ± 0.24	
			3000	NA	47.2 ± 1.6	35.2 ± 1.6	0.259 ± 0.030	98.9	79.7 ± 14.2	47.4 ± 15.6	447± 177	NA	4.05 ± 0.35	
			10,000	NA	48.0 ± 2.1	35.9 ± 1.6	0.255 ± 0.031	99.2	78.8 ± 11.9	46.6 ± 12.5	419 ± 259	55 ± 12	3.94 ± 0.35**	
Sprague-Dawley rats†	MON 0818 (POEA)	F1	0	4.99/3.09	43.0 ± 1.93	33.1 ± 1.85	NR	99.4	83 ± 8.5	NR	NR	78.9 ± 12.12	4.2 ± 0.38	Knapp 2007
			100	5.00/3.11	42.9 ± 2.10	32.8 ± 1.47	NR	99.2	79 ± 10.3	NR	NR	79.4 ± 8.95	4.4 ± 0.55	
			300	5.03/2.90*	43.7 ± 1.61	33.5 ± 1.13	NR	99.3	84 ± 7.4	NR	NR	78.0 ± 8.75	4.2 ± 0.54	
			1000	5.09/3.03	42.8 ± 1.53	33.0±0.71	NR	98.9	77 ± 12.5	NR	NR	71.2 ± 13.28	4.2 ± 0.50	

Note. *Statistically different from control, $p < .05$; **statistically different from control, $p < .01$. #, Average glyphosate intake in the 1000-, 3000-, and 10,000-ppm groups during the premating period reported to be 99, 293, and 985 mg/kg/d, respectively, for F_0 males; 104, 323, and 1054 mg/kg/d, respectively, for F_0 females; 117, 352, and 1161 mg/kg/d, respectively, for F_1 males; and 123, 371, and 1218 mg/kg/d, respectively, for F_1 females. †Average POEA intake in the 100, 300 and 1000 ppm groups during the premating period reported to be 6, 18, and 60.5 mg/kg/d, respectively, for F_0 males and 7, 20.7, and 70.4 mg/kg/d, respectively, for F_0 females; NR = not reported.

(3/gender/litter) were weaned on PND 21/22, and then were administered POEA in the diet at mg/kg/d target doses equal to the mean POEA intake of the F_0 generation animals until PND70. At PND70, F_1 animals selected for breeding in the control and high-dose groups (2/gender/litter) were administered 0 or 1000 ppm POEA, respectively, in the diet either through mating (males) or mating, gestation, and lactation (females). The majority of experimental parameters evaluated in this study were unaffected by treatment, including survival and clinical condition, body weight and food consumption, reproductive performance, organ weights, macroscopic and microscopic morphology of the F_0 and F_1 parental generations; clinical condition and body weights, anogenital distance, preputial separation and vaginal opening, estrous cyclicity, spermatogenic endpoints, testosterone and thyroid hormone levels of the F_1 generation; and clinical condition, body weights, litter viability and postnatal survival of the F_2 litters. In the F_0 high-dose group, a significant increase was observed in the mean number of implantation sites that could not be accounted for by resorptions or pups born (live or dead). This finding was accompanied by a reduced mean number of pups born and a decreased live litter size in the high dose group. Three dams in the high dose group had litters of only two to four pups each, two of which showed total litter loss by PND4; this finding contributed to lower PND4 postnatal survival at the high dose. Upon breeding of the F_1 generation, however, none of these findings was reproducible. Because the observed changes were not reproducible between generations, and in some cases were not considered statistically significant, these findings were considered equivocal. Knapp (2007) considered 300 ppm (approximately 20 mg/kg/d) to be the NOAEL for reproductive and developmental toxicity in this screening study.

Knapp (2008) conducted a second reproductive/developmental screening study according to the OECD 422 test guideline and using two different POEA surfactants. Groups of Sprague-Dawley rats (12/gender/group) were administered either the same POEA from the 2007 study (POEA 1) at a concentration of 1000 ppm in the diet (to assess whether the equivocal litter effects seen at the high dose in the 2007 study could be repeated) or another POEA surfactant (POEA 2) at concentrations of 0, 30, 100, 300, or 2000 ppm in the diet. Treatment was administered from 14 d prior to mating for up to 72 d, including during gestation and lactation in the females. POEA 1 intake during the premating period averaged 76 mg/kg/d in males and 86 mg/kg/d in females administered 1000 ppm in the diet. POEA 2 intake during the premating period averaged 2, 8, 23, and 134 mg/kg/d for males and 3, 9, 26, and 148 mg/kg/d for females in the 30-, 100-, 300-, and 2000-ppm groups, respectively. In the group administered 1000 ppm POEA 1, all animals survived to scheduled necropsy with the exception of 1 female with dystocia that died on PND1 and a second female euthanized with a ruptured uterus on GD30. Because dystocia was noted in one of the F_1 control-group females in the 2007 study, this finding was not considered treatment related. Parental systemic toxicity (mean body weight losses, lower mean body weight gains and reduced food consumption for both genders) was observed with POEA 2 at 2000 ppm, but not in any other treatment group. No treatment-related effects were observed on any reproductive parameters assessed, organ weights, or macroscopic and microscopic histology. The mean number of implantation sites was reduced and the number of unaccounted-for sites increased at 2000 ppm of POEA 2. Further, the mean live litter size on PND 0, mean number of pups born, and postnatal survival to PND4 were reduced in this treatment group. Compared to the control, mean pup weight on PND1 was also quantitatively reduced. Based on these findings, Knapp (2008) considered the NOAEL for POEA 2 to be 300 ppm (approximately 23 mg/kg/d). With regarding to the repeat study with POEA 1, no test substance-related signs of systemic toxicity, reproductive effects, or effects on pup survival or morphology were noted at any dose; thus, Knapp (2008)

considered the NOAEL for this POEA to be 1000 ppm (approximately 81 mg/kg/d).

Reproductive/Developmental Data From Other Animal Studies

Glyphosate. The National Toxicology Program (NTP) conducted a 13-wk glyphosate feeding study in F344/N rats and B6C3F$_1$ mice (Chan and Mahler 1992). Groups of 10 male and 10 female rats and mice were administered feed containing 0, 3125, 6250, 12,500, 25,000, and 50,000 ppm glyphosate for 13 wk. Average glyphosate consumption in the 3125-, 6250-, 12,500-, 25,000-, and 50,000-ppm groups was 205, 410, 811, 1678, and 3393 mg/kg/d, respectively, for males and 213, 421, 844, 1690, and 3393 mg/kg/d, respectively, for females. An additional 10 animals per gender 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 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 significantly 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 6). Mean body weight of female mice in the highest glyphosate treatment group (50,000 ppm) was also numerically 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 marked 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

TABLE 6. Reproductive Endpoints Assessed in a 13-wk Glyphosate Feeding Study in Mice and Rats (Chan and Mahler 1992)

Animal model (number per group)	Dose (ppm)	Exposure	Terminal body weights (g)^	Left epididymal tail weight (g)	Left testes weight (g)	Left epididymis weight (g)	Sperm concentration (10^6)	Sperm motility (%)	Spermatid count (mean/10^{-4} ml)	Spermatid heads (10^7/testis)	Spermatid heads (10^7/g testis)	Estrous cycle length (d)
F344/N rats (10 M, 10 F)	0	Daily, 13 wk	385 ± 5	0.170 ± 0.004	1.54 ± 0.03	0.448 ± 0.007	610 ± 36	91 ± 1	70.15 ± 3.00	14.03 ± 0.60	9.10 ± 0.35	4.90 ± 0.10
	12500	Daily, 13 wk	350 ± 5	0.168 ± 0.006	1.52 ± 0.05	0.437 ± 0.016	561 ± 23	92 ± 1	65.33 ± 5.49	13.07 ± 1.10	8.48 ± 0.64	5.00 ± 0.07
	25000	Daily, 13 wk	340 ± 5*	0.167 ± 0.004	1.56 ± 0.03	0.440 ± 0.004	485 ± 23**	92 ± 2	67.23 ± 2.05	13.45 ± 0.41	8.63 ± 0.30	4.90 ± 0.10
	50000	Daily, 13 wk	305 ± 7**	0.179 ± 0.006	1.56 ± 0.02	0.452 ± 0.007	486 ± 23**	91 ± 1	69.00 ± 1.71	14.06 ± 0.35	9.04 ± 0.20	5.40 ± 0.21*
B6C3F1 mice (10 M, 10 F)	0	Daily, 13 wk	32.0 ± 1.0	0.015 ± 0.001	0.110 ± 0.002	0.044 ± 0.001	1162 ± 44	91 ± 1	67.20 ± 2.30	2.15 ± 0.07	19.61 ± 0.92	4.06 ± 0.05#
	12500	Daily, 13 wk	31.9 ± 0.9	0.014 ± 0.001	0.111 ± 0.003	0.043 ± 0.002	1370 ± 130	91 ± 1	63.18 ± 3.06	2.02 ± 0.10	18.17 ± 0.71	4.00 ± 0.00
	25000	Daily, 13 wk	29.4 ± 0.7*	0.014 ± 0.001	0.111 ± 0.002	0.044 ± 0.001	1189 ± 60	92 ± 1	61.93 ± 1.92	1.98 ± 0.06	17.87 ± 0.60	4.00 ± 0.00
	50000	Daily, 13 wk	27.2 ± 0.4**	0.014 ± 0.001	0.110 ± 0.003	0.042 ± 0.001	1308 ± 97	89 ± 1	65.40 ± 2.89	2.09 ± 0.09	18.99 ± 0.73	4.00 ± 0.00#

Note. Average glyphosate consumption in the 3125-, 6250-, 12,500-, 25,000-, and 50,000-ppm groups was 205, 410, 811, 1678, and 3393 mg/kg/d, respectively, for males and 213, 421, 844, 1690, and 3393 mg/kg/d, respectively, for females. ^, Terminal body weights are mean values for males only. *Statistically different from control, $p \leq .05$; **statistically different from control, $p \leq .01$. #, $n = 9$.

observed in male rats treated at the two highest glyphosate concentrations. In addition, 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 Chan and Mahler (1992), 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 7). The study consisted of a 6-wk preliminary period in which no treatment was provided, followed by a 6-wk treatment period, and finally a 6-wk recovery period in which treatment was discontinued. Groups of 4 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. Further, 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, percent 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 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 animals were not sham-handled, then the effects of treatment may be stress related. Based on statistical analyses, Yousef et al. (1995) were not able 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

TABLE 7. Overall Mean Semen Characteristics of New Zealand White Rabbits Treated Orally With A Glyphosate-Based Formulation (Yousef et al. 1995)

Dose	Body weight (kg)	Semen volume (ml)	Sperm conc. ($\times 10^4$/cc)	Abnormal sperm (%)	Dead sperm (%)	Methylene blue reduction time (min)	Initial fructose (mg/100 ml)	Semen osmolality
Pretreatment period								
0	2.944 ± 0.030	0.88	264	9.4	6.6	5.07	337	248
1/100 LD_{50}	2.979 ± 0.060	0.83	265	9.7	6.4	5.22	324	255
1/10 LD_{50}	3.173 ± 0.050*	0.88	262	10.3	6.5	5.07	336	253
Treatment period								
0	3.008 ± 0.020	0.83	413	12.5	8.9	3.53	359	283
1/100 LD_{50}	2.811 ± 0.060*	0.6*	242*	21.9*	19.5*	6.54*	281*	252*
1/10 LD_{50}	3.125 ± 0.030	0.62*	262*	22.6*	21.4*	7.26*	267*	261*
Recovery period^								
0	3.108 ± 0.010	0.82	596	20.4	4.1	3.48	312	278
1/100 LD_{50}	2.816 ± 0.070*	0.68*	473*	25.7*	6.2	5.0*	298	284
1/10 LD_{50}	3.368 ± 0.020*	0.73*	467*	24.1*	7.5*	5.29*	297	278

Note. ^, Data from only three animals per group were recorded for the recovery period. *Significantly different from control, $p < .05$.

of glyphosate treatment. In addition, the rabbits used in the study were quite small (approximately 3 kg); buck rabbits used for mating purposes are usually larger, often weighing 4–5 kg. This suggests the animals may not have been fully 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 not possible to draw conclusions regarding the effects of glyphosate treatment on male rabbit fertility.

Commercial herbicide formulations. Dallegrave et al. (2007) conducted a 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 8 and 9). Sixty pregnant female Wistar rats (15/group) were gavaged daily with 0, 50, 150, or 450 mg/kg/d "glyphosate-Roundup" throughout pregnancy and lactation. As previously discussed in reference to an earlier study by 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 d of age) as well as at the age of adulthood (140 d of age) to investigate systemic and reproductive effects of treatment. For the females, sacrifices occurred on the first estrus after 65 or 140 d of age. Organ weights were recorded relative to total body weights. Additional investigations were also conducted on males. The numbers of

TABLE 8. Reproductive Outcome and Sexual Development Data for Rats Treated With Roundup Through Pregnancy and Lactation (Dallegrave et al. 2007)

Animal model (number per group)	Exposure	Dose (mg/kg/d)	Maternal relative weight gain (%)	Number of live fetuses (number per litter)	Number of PD0 males/ females	Mean offspring weight at birth (g)	Males		Females
							Day of testis descent	Day of preputial separation	Day of vaginal opening
Wistar rats (15)	Through pregnancy and lactation	0	36.8	160 (10.7)	75/85	5.77	15.0	31.7	34.9
		50	38.7	161 (10.8)	80/81	5.97	15.0	31.7	37.6*
		150	38.8	165 (10.3)	83/82	5.90	15.0	31.5	36.9*
		450	40.9	162 (10.8)	93/69	6.05	14.8	30.7*	36.7*

Note. *Significantly different from control, $p < .05$.

TABLE 9. Effects of In Utero and Lactational Roundup Exposure on Sperm Production-Related Parameters (Dallegrave et al. 2007)

Dose (mg/kg/d)	Daily sperm production ($\times 10^6$)	Number of sperm ($\times 10^6$)	Sperm transit rate (d)	Abnormal sperm (%)	Tubules with spermatogenesis (%)	Tubule diameter (μm)	Testosterone level (ng/ml)
Puberty (65 d of age)							
0	11.1	44.2	4.1	8.6	84	166.7	5.2
50	12.2	53.9	4.6	16.7*	77	159.6	4
150	12.1	67.2	5.3	9.2	78.7	159.6	3.2
450	11.7	57.4	5.1	11.6	75	160.4	1.5*
Adulthood (140 d of age)							
0	20.5	344.7	17.7	5.4	92	180.8	3.9
50	15.3*	251.0*	17.5	8.3	73.5	187.3	3.4
150	19.7	368.7	20.2	8.4	74.5	173.4	6.3
450	14.7*	257.1*	18.5	7.7	65	185.1	3.3

Note. *Significantly different from control, $p < .05$.

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 spermatids per animal by 6.1 d, 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 significant effects on the female offspring were observed. Male offspring at puberty exhibited a statistically increased percentage of abnormal sperm at the low but not 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 were a true finding. Further, the effect on testosterone levels was no longer evident at the age of adulthood. In adulthood, daily sperm production and sperm number were also 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, 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 were noted. 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 investigators fail to mention in their report. This obvious omission suggests that Dallegrave et al. (2007) 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. In addition, none of the guideline-compliant reproductive studies nor the NTP 13-wk subchronic study discussed previously—all of which involved much greater glyphosate exposures—reported such testicular anomalies.

Two non-guideline studies were conducted using Herbicygon, a commercial herbicide formulation containing glyphosate; no other formulation information was 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 both in dams exposed to Herbicygon via drinking water and in 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 not possible to know exactly how much glyphosate the treated rats received in these experiments. Further, 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 2 wk 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 feed 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 80.7 and 52.79 g in the 0.5% and 1% treatment groups, respectively, versus 92 g 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 g versus 92 g 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 study. 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. In addition, 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. Several investigators found that food restriction (in the absence of toxicants) affects the activity of many enzymes, including those examined in this study (Boll et al. 1996; Goodridge et al. 1996; 1998; Martin et al. 1990; Martins et al. 1985; 1986; Nagy et al. 1978; Sachan and Das 1982; Sassoon et al. 1968; Xie et al. 1995). Daruich et al. (2001) did 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 halfway through gestation; thus, although data show that the period of restricted diet was sufficient to affect total maternal weight gain and liver weights, it may not have been sufficient 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 earlier. It should be noted that Beuret et al. (2004) incorrectly referred to glyphosate as an organophosphate 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 (SOD) 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 g versus 92 g for controls). Liver weights were also 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 whether 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 those 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 elevated with treatment in the fetuses (13.28 ± 0.58 μmol NADPH/min/g tissue versus 9.03 ± 1.01 in controls), but not in dams. Liver catalase and SOD activity levels were not affected by treatment. It is not possible to draw any conclusions regarding the effects of Herbicygon treatment from these data because no restricted diet controls were included in the study. Other studies showed that dietary restriction may affect lipid peroxidation and glutathione peroxidase activity levels (Kim et al. 1996; Mura et al. 1996; Rao et al. 1990); therefore, it is not known whether 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 a surfactant) cannot 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.

Romano et al. (2010) conducted an experimental investigation regarding preputial separation in male Wistar rats using Roundup Transorb containing 480 g/L glyphosate. Groups of newly weaned male rats (n = 16–18) were gavaged daily from PND23 to PND53 with 0, 5, 50, or 250 mg/kg/d of Roundup Transorb. It is not known whether the rats in each group were taken from the same or different litters to control for potential litter effects. Rats were evaluated daily for balanopreputial separation beginning on PND 33. On PND 53, the rats were sacrificed, the testes and adrenal glands were weighed and examined both histologically and morphometrically, and serum hormone assessments were conducted for testosterone, estradiol and corticosterone. Although data were not shown, no marked effects of treatment on body weights were reported. Daily exposure to Roundup Transorb was reported to be associated with a significant delay in the time of preputial separation in the mid- and high-dose groups; body weight at preputial separation, however, was unaffected. It should be noted, however, that the age of preputial separation reported for the control rats (37 d) is extremely early compared to that reported in various other studies (40–43 d of age). Further, Romano et al. (2010) did not report whether the assessment was conducted blinded, whether the observations were made at the same time each day, or whether signs of incomplete separation or persistent threads were observed at any time. The extremely early age of preputial separation reported in this study suggests that the investigators may not have distinguished incomplete from complete separation. Thus, these data are likely unreliable.

Relative testicular weights were increased at the high dose and relative adrenal weights were elevated in both the mid-dose and high-dose groups. It should be noted, however, that these data were highly variable. Although no abnormal pathology was reportedly observed in these tissues, the seminiferous epithelial

height was reduced in a dose-related manner and the luminal diameter of the seminiferous tubules increased in all treatment groups compared to controls. The micrographs presented in support of these findings are of poor quality, exhibiting numerous fixation artifacts (e.g., shrinkage) that prevent an accurate estimation of tubule lumen diameter or seminiferous epithelial cell height. Furthermore, the spermatogenic cycle does not stabilize and become synchronous for some time after puberty; thus, the findings observed may have simply been due to maturational variation among individual tubules in the peripubertal rat. Thus, the morphometric analyses conducted are unreliable and confounded by tubules being at different stages of maturation. Finally, Romano et al. (2010) report a dose-related reduction in serum testosterone levels compared to controls, with testosterone levels at the high dose being approximately 50% lower than control; serum estradiol and corticosterone concentrations were unaffected. The reduced testosterone concentrations are in contradiction to the reported rise in testicular weights. In conclusion, the results of this study lack the scientific rigor necessary to support a definitive scientific conclusion and do not offset the findings of previous large, definitive, and GLP-compliant studies concluding that Roundup and glyphosate do not adversely affect reproductive development.

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 Practices (GLP) protocols and/or according to established testing guidelines to indicate that glyphosate, POEA surfactants, or commercial glyphosate herbicides are developmental or reproductive toxicants. While a few studies claimed adverse reproductive or developmental effects (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 (1) have used commercial herbicide formulations rather than pure glyphosate or surfactant and have not followed up with additional studies to determine if findings were due to the pesticide active ingredient or another formulation component, (2) have failed to include appropriate controls, (3) have used inadequate numbers of animals per treatment group, and (4) have not clearly stated 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 GLP protocols and/or according to established testing guidelines found no marked effects of treatment on reproduction or in offspring, despite significant toxicity in treated dams (Holson 1990; 1991; IRDC 1980b; Moxon 2000; Reyna 1990; Schroeder 1981). 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 3500 mg/kg/d 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 1000 mg/kg/d for studies required by regulatory agencies. In conclusion, 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 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.

Cell Cycle/Transcriptional Inhibition Studies Several studies were performed to ascertain whether glyphosate is likely to inhibit cell cycle progression and transcription (Table 10). These studies were conducted 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. In addition, 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 study, the advantages of the use of the sea urchin as a biomarker of toxicity were discussed; 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) 3 min after appearance of the fertilization membrane. It should be noted that this concentration of glyphosate is 34-fold greater than the allowable maximum contaminant level of glyphosate in drinking water (U.S. EPA 2009a). 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, that (1) Roundup formulations contain surfactants; (2) the observations are consistent with the effects of a surfactant; and (3) 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 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 m*M* exerted no marked effect on cell division, adding progressively larger amounts of glyphosate to 0.2% Roundup (which already contains 2 m*M* glyphosate) induced the delay in cell division. The data to support these claims, however, are weak. In particular, when glyphosate was added at increasing concentrations, no dose-response relationship was evident. Further, 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, Marc et al. (2002) 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 nontoxic 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 by Marc et al. (2003). Data demonstrated that 0.8% Roundup (glyphosate concentration

TABLE 10. Sea Urchin Embryo Assays Assessing the Ability of Glyphosate and Glyphosate-Based Formulations to Inhibit Cell Cycle Progression and Transcription

Study	Basic experimental design	Findings
Medina et al. 1994	20 µl suspension of fertilized sea urchin eggs exposed to 480 g/L Roundup (containing 1.4×10^{-4} M glyphosate) 3 min after appearance of the fertilization membrane; eggs observed for ~24 h until the pluteus (free-swimming larvae) stage.	Roundup-treated eggs exhibited deformed or destroyed nuclear elements, as well as a perforated nuclear membrane. *Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, concentrations of Roundup used in this study are not environmentally relevant.*
Marc et al. 2002	Effects of 0.8% Roundup (containing 8 *M* glyphosate), 8mM pure glyphosate, and 0.2% Roundup supplemented with concentrations of glyphosate up to 10 mM, on the 1st cell division in sea urchin embryos were investigated; ~100 embryos were scored per treatment group. The kinetics of CDK/cyclin B activation were also measured using H1 protein as a substrate.	Roundup exposure was associated with an increase in first cell division delay, and inhibits CDK/cyclin B activation; 8 m*M* glyphosate had no impact on urchin cell division; supplemental glyphosate added to 0.2% Roundup induced cell division delay, but no dose-response relationship was observed. *Concentrations of Roundup and glyphosate used in this study are not environmentally relevant.*
Marc et al. 2003	Impact of 0.8% Roundup (containing 8 *M* glyphosate) exposure on CDK/cyclin B activation at selected times following fertilization (≤120 min) investigated using H1 histone as a phosphorylation substrate.	Authors report that Roundup blocked CDK/cyclin B activation, but urchins underwent cell division, albeit delayed. *Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, concentrations of Roundup used in this study are not environmentally relevant.*
Marc et al. 2004a	Effect of various concentrations of glyphosate-containing herbicides (Roundup3Plus, Amega, Cargly, Cosmic, and Roundup Biovert) on time of 1st cell division postfertilization assessed. Glyphosate concentrations of herbicide preparations tested ranged from 0.1–30 m*M*.	All herbicides tested delayed the 1st cell division in a dose-dependent manner, but response across herbicides was independent of glyphosate concentration. *Because herbicidal formulations were tested, findings cannot be specifically attributed to glyphosate. Also, herbicide concentrations used in this study are not environmentally relevant.*
Marc et al. 2004b	Whether 10 mM Roundup inhibits CDK/cyclin B activation by preventing dephosphorylation of CDK1/cyclin B tyrosine 15 complex was examined using affinity purification and Western blot analysis; cells were examined at the time of 1st cell division postfertilization. The effects of 10 m*M* pure glyphosate and Roundup 3plus (at a concentration equivalent to 10 m*M* glyphosate) on phosphatase activity of the cdc25C recombinant protein and embryo extracts were also assessed.	Roundup 3Plus exposure caused a 30 min delay in CDK1 tyrosine phosphorylation and was associated with a 70% inhibition of DNA synthesis; neither 10 m*M* pure glyphosate nor the concentration of Roundup 3Plus (containing 10 m*M* glyphosate) was associated with a change in cdc25C protein. *Because only the herbicidal formulation was tested for effects on CDK1 phosphorylation, the findings cannot be specifically attributed to glyphosate. Also, concentrations of glyphosate and Roundup used in this study are not environmentally relevant.*
Marc et al. 2005	Impact of 0.2%, 0.4 %, and 0.6% Roundup (containing 2, 4, and 6 m*M* glyphosate), 30–900 mg/L POEA, and 0.2% Roundup with 8 m*M* supplemental glyphosate on the percentage of embryos hatching, and the delay in hatching time was observed with phase contrast microscopy. Transcriptional activity of embryo suspensions exposed to 0.2%, 0.4%, and 0.6% Roundup quantified by incorporation of 5-[^{3}H]-uridine. Expression of sea urchin hatching enzyme mRNA (SgHE) in urchins exposed to 1% Roundup (10 m*M* glyphosate) measured by RT-PCR.	0.2-0.6% Roundup was associated with a dose-dependent decrease in percentage of embryos hatching, and an increase in hatching delay; addition of 8 m*M* glyphosate to 0.2% Roundup increased the hatching delay (but no statistics are provided to show that this is significant) and 8 m*M* glyphosate alone had no effect; a dose-dependent decrease in urchin embryo transcription seen with 0.1–0.8% Roundup; 30–900 mg/L POEA led to irreversible embryonic damage or lethality. *Concentrations of Roundup and glyphosate used in this study are not environmentally relevant. Also, data suggest POEA, not glyphosate, is responsible for adverse effects of Roundup to sea urchin embryos.*

of 8 m*M*) 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 min), 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 Marc et al. (2003) claimed 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. Further, although the sea urchin embryos treated with Roundup were stated not to undergo CDK1/cyclin B activation, enough activation was apparently present to induce cell division, albeit delayed. In addition, using an assay to examine protein synthesis via incorporation of radiolabeled methionine, Roundup appeared to decrease protein production during the first 2 h, which could potentially inhibit or delay various reproduction processes. In the last set of data presented, Marc et al. (2003) examined cyclin B abundance and phosphorylation status at 60 and 75 min after fertilization using an antibody detection method. As shown previously (Marc et al. 2002), Roundup did not significantly affect CDK1/cyclin B activation. Overall, data presented in this study did 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 glyphosate concentrations of the herbicide preparations tested ranged from 0.1 to 30 m*M*. The percentage of embryos undergoing the first postfertilization cell division was assessed by phase microscopy at 60-min intervals up to 300 min postfertilization. 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 m*M* glyphosate, after which CDK1/cyclin B complex was affinity-purified from embryo extracts at 10-min intervals postfertilization. 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 expression against that of another protein that is more commonly used for such purposes was not evaluated. Considering that these experiments were designed to examine effects on CDK1/cyclin B activation, the use of CDK1 expression as a loading control seems highly inappropriate. From this experiment, Marc et al. (2004b) reported that herbicide treatment delayed the cyclin B pattern changes associated with activation of CDK1 during the first postfertilization cell division. Furthermore, treatment delayed CDK1 tyrosine phosphorylation by 30 min 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 m*M* glyphosate and Roundup 3plus (at a concentration equivalent to 10 m*M* 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 postfertilization. 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 Marc et al. (2004b) did not present data using pure glyphosate in the DNA synthesis experiment is interesting, especially since glyphosate was purportedly used 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 study, Marc et al. (2005) examined the influence 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 reverse-transcription polymerase chain reaction (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, or 6 m*M* of Roundup (30 replicates per concentration). The morula stage was chosen because previous studies showed that Roundup delayed the first cell divisions (Marc et al. 2002; 2003), and thus, experiments focused on the impact of Roundup on later cell divisions and transcription. Interestingly, the positive control agent was applied 10 min 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 concentration-related decrease in percent embryos hatching after Roundup 3plus treatment was observed. The delay in hatching time due to administration of 8 m*M* pure glyphosate, 0.2% Roundup, and 0.2% Roundup supplemented with 8 m*M* glyphosate was also measured. Test agents were again administered during the morula stage (four trials per treatment group). Pure glyphosate delayed hatching by 33 $\pm$ 6 min, 0.2% Roundup resulted in a 128 $\pm$ 30 min inhibition, and the 0.2% Roundup plus 8 m*M* glyphosate supplementation resulted in a delay of 205 $\pm$ 30 min. Thus, although glyphosate alone exerted little effect, co-administration of additional glyphosate with Roundup increased hatching delay time. Marc et al. (2005) 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 $\leq$8 m*M* exerted no marked effect on hatching time. The four glyphosate-containing formulations (Roundup Biovert, Roundup 3plus, Cargly, and Cosmic), however, all produced delays in hatching, although some formulations were more potent than others. Despite the lack of effect with neat glyphosate, Marc et al. (2005) concluded that glyphosate might 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. Further, SgHE mRNA expression was reduced for 2 h postfertilization; however, the results of only a single experiment were shown to support this claim and no statistical analyses were conducted. Overall, these experiments demonstrated that glyphosate-based herbicidal formulations impact cell divisions in the sea

urchin embryo; however, data 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) was conducted that did not address glyphosate, but rather examined the toxicity of three commonly used mild surfactants in sea urchins. The results of this study provide support that the effects observed in sea urchin embryos following herbicide application are due to a component of the formulations rather than to 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 surfactants tested produced inhibition of cleavage at concentrations lower than those commonly used in consumer products. In addition, 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 herbicidal formulations containing similar surfactants should be considered unremarkable.

Summary—Cell Cycle/Transcriptional Inhibition Studies Overall, results using the sea urchin model showed that exposure to high concentrations of glyphosate-based herbicide formulations and substances used on consumer products 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 $\geq$8 m*M* glyphosate were used in these studies (Medina et al. 1994; Marc et al. 2002; 2003; 2004a; 2004b; 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). Further, 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 lacking. Finally, there is not sufficient 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 might produce 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 low concentrations of surfactants and detergents. This is noted in the *U.S. EPA Endocrine Disruptor Screening*

TABLE 11. Mechanistic Studies Assessing the Potential Endocrine-Disrupting Effects of Exposure to Glyphoste and Glyphosate-Based Formulations

Study	Basic experimental design	Findings
Petit et al. 1997	Recombinant yeast system expressing the estrogen receptor (ER): Estrogenic potential of various chemicals, including 10^{-8} to 10^{-4} *M* glyphosate, tested in yeast cells expressing the rainbow trout ER linked to a *lacZ* reporter gene; cells treated to test agents for 4 h.	Glyphosate did not demonstrate estrogenic activity.
Lin and Garry 2000	Estrogen-responsive MCF-7 cells: Response of MCF-7 cells to Roundup or glyphosate exposure assessed; cell proliferation after a 7-d exposure period in presence and absence of steroid growth factor-deficient FBS examined by flow cytometry; cell viability and apoptosis examined after 72 h of incubation by flow cytometry and propidium iodide.	Cell proliferation increased with exposure to both Roundup and glyphosate, but response was similar with and without FBS, suggesting it was mediated through a nonestrogenic pathway; no cytotoxicity or apoptosis observed due to glyphosate exposure.
Meulenberg 2002	Displacement of estradiol (E_2) from human sex hormone binding globulin (SHBG): Displacement of tritiated E_2 from SHBG by different concentrations of various test agents (including glyphosate) measured in vitro.	Glyphosate reported to have shown ambiguous results for E_2 displacement from SHBG.
Xie et al. 2005	Rainbow trout vitellogenin assay: Ability of 0.11 mg/L glyphosate and other herbicides to induce vitellogenin expression in trout assessed.	Glyphosate was not found to have estrogenic activity in this assay.
Kojima et al. 2004	Human ERα, ERβ, and androgen receptor (AR) binding: More than 200 pesticides were tested for agonist or antagonist activity at human ERα, ERβ, and AR transfected into Chinese hamster ovary cells; $\leq 10^{-5}$ *M* glyphosate tested.	Glyphosate was not noted to affect hormone binding in any of the receptor subtypes tested.
Walsh et al. 2000	Steroidogenic acute regulatory (StAR) protein synthesis: Impact of Roundup (with 180 g/L glyphosate) and other herbicides on steroidogenesis in MA-10 Leydig tumor cells was assessed by measuring progesterone production by radioimmunoassay; levels of StAR mRNA assessed using Northern blots.	20–100 μg/ml Roundup, but not pure glyphosate, caused a significant dose-dependent decrease in progesterone production; 25 μg/ml Roundup did not influence overall protein levels, but decreased levels of StAR mRNA.
Levine et al. 2007	Inhibition of progesterone production in MA-10 mouse Leydig cells: MA-10 cells were exposed for 2 h to Roundup with and without glyphosate, as well as to various surfactants; the hCG-stimulated increase in progesterone production was measured following incubation; impact of surfactants on StAR protein levels was assessed by Western blot on hCG-stimulated and nonstimulated MA-10 cells; impact of treatment on mitochondrial membrane function was determined by JC-1 cationic dye.	Exposure to surfactants, as well as to Roundup with and without glyphosate, was associated with a decrease in hCG-progesterone production, decreased expression of the StAR protein, and a decrease in mitochondrial membrane function.
Richard et al. 2005	Aromatase activity and mRNA levels in JEG3 cells and placental and equine testicular microsomes: Aromatase activity in JEG3 cells treated 1 and 18 h with 0.2–2% Roundup (or corresponding concentrations of glyphosate) measured by radioimmunoassay; aromatase mRNA expression measured by RT-PCR. Aromatase activity in microsomes from full-term placentas and equine testes also assessed upon 15 min exposure to Roundup or glyphosate.	JEG3 cells: 0.2–2% Roundup has significantly greater impact on cell viability than glyphosate of corresponding concentrations; aromatase activity significantly increased at 1 h and significantly decreased at 18 h after exposure to 0.01% Roundup; aromatase mRNA also decreased at 18 h following Roundup exposure; $\leq$0.8% glyphosate for 1 or 18 h had no effect on aromatase activity. Microsomes: Aromatase activity decreased at $>$0.05% Roundup and $>$0.5% glyphosate. *Concentrations of Roundup and glyphosate used in this study are not environmentally relevant.*

(*Continued*)

TABLE 11. (*Continued*)

Study	Basic experimental design	Findings
Benachour et al. 2007	Aromatase activity in JEG3 and human embryonic kidney 293 cells and placental and equine testicular microsomes: Cell viability and aromatase activity following 1, 24, or 48 h of treatment with 1–2% Roundup or equivalent concentrations of glyphosate assessed as above; cultures treated in either serum-containing or serum-free media.	293 Cells were more sensitive than JEG3 cells; cells in serum-free media were more sensitive than those in serum-containing media; Roundup was substantially more cytotoxic than glyphosate; Roundup decreased aromatase activity in microsomes in temperature-responsive manner. *Concentrations of Roundup and glyphosate used in this study are not environmentally relevant. Also, the pH values of the test agents were not adjusted appropriately.*
Gasnier et al. 2009	Aromatase activity and anti-estrogenicity in HepG2 cells and anti-androgenicity in MDA-MB-453-kb2 cells: Aromatase activity following 24 h of treatment with glyphosate or 1 or 4 herbicide formulations; anti-estrogenicity and anti-androgenicity assessed following 24 h of treatment with same test compounds; incubations done in serum-free media.	Herbicide formulations inhibited aromatase activity and exhibited dose-dependent antiestrogenic and antiandrogenic activity; results were not proportional to glyphosate concentration of formulations; ≤0.3% glyphosate has no effect on aromatase or estrogenic activity; androgenic activity altered by glyphosate, but not in dose-dependent manner. *Results with glyphosate alone suggest no endocrine modulating activity. Results with formulations confounded by presence of surfactants and other ingredients.*
Hokanson et al. 2007	Gene expression in MCF-7 cells: Gene expression following 18 h of exposure to 0.001–0.1% of a glyphosate-containing herbicide was assessed by DNA microarray and RT-PCR.	Treatment altered gene expression, but of seven genes selected for further study, disregulation was confirmed by RT-PCR for only three. *Because a herbicidal formulation was tested, findings cannot be specifically attributed to glyphosate. Also, no evidence indicates that these changes were mediated through endocrine-disruption.*
Paganelli et al. 2010	Neural crest cell marker expression in *Xenopus laevis* embryos: Expression of various neural crest cell markers following exposure of 2-cell stage embryos to 1/5000–1/3000 dilutions of Roundup Classic or injection with 500 pg glyphosate.	Treatments reduced neural crest cell marker expression and appeared to be associated with cranial malformations; possible involvement of retinoic acid pathway hypothesized. *Glyphosate solution was not pH-adjusted and was injected into embryos, making relevance to environmental exposures questionable.*

Program Test Guideline OPPTS 890.1200: Aromatase (Human Recombinant) (U.S. EPA 2009b), which clearly warns that all glassware and apparatus used in the microsome preparations need to be free of detergent residue. Furthermore, if detergent residues compromise study viability testing, measurable concentrations of detergent-like substances would certainly overload such in vitro systems, and thus do not represent a viable approach to investigating endocrine disruption.

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 2 estrogen-responsive elements were treated in culture at 10^{-8} to 10^{-4} *M* of each test agent for 4 h. 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 exerted no marked effect on the basal level of β-galactosidase activity. Only those test

agents shown to be positive for estrogenicity in the yeast system, plus 11 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 might 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-h incubation, the cells were then treated with different dilutions of test chemicals, 10^{-9} *M* estradiol (positive control), or solvent vehicle (negative control). After 7 d in culture, cell numbers and viability of harvested cells were assessed using a fluorescence-activated cell sorter. In separate experiments, cytotoxicity (following 72-h 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 found 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. Maximal 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 7-d proliferation studies. In addition, neither glyphosate nor "Roundup" was found 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.

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 affect the free concentrations of various sex hormones. Because it is assumed that only the free fraction of such hormones exerts biological activity, such changes may 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 h of 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 possess a threefold greater affinity for SHBG than E_2, testosterone 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. Meulenberg (2002) indicated that glyphosate demonstrated ambiguous results for displacement of E_2 from SHBG, although actual experimental data were not shown. Because no data were presented for independent review, conclusions on whether glyphosate affects the ability of SHBG to bind sex hormones in the blood cannot be made.

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 (VTG) assay. In fish, adult female production

of VTG is mediated by estrogenic activity; thus, VTG 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 fish for 7 d to 0.11 mg/L glyphosate exerted no significant effect on VTG levels, suggesting that glyphosate is unlikely to alter estrogenic activity.

Kojima et al. (2004) tested more than 200 pesticides for their ability to act as agonists and antagonists to 2 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 3 h of transfection, cells were incubated for 24 h with varying concentrations of test agent. To assess antagonistic activity to hERα, hERβ, and hAR, the appropriate transfected cells were co-incubated with test agent and 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 producing 20% inhibition of activity of 10^{-11} *M* E_2, 10^{-10} *M* E_2, or 10^{-10} *M* DHT at the hERα, hERβ, and hAR, respectively. Although not completely clear from the methods section of the paper, it appears that Kojima et al. (2004) 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 demonstrated greater activity than the REC_{20} or RIC_{20}, respectively. The values presented in the study are the mean and standard deviations derived from at least three independent experiments. Although glyphosate was tested, it was not identified as a chemical possessing agonist or antagonist activity at any of the three receptor sites evaluated. It is noteworthy that specific tests for cell toxicity were not conducted, although assays were conducted at concentrations $\leq 10^{-5}$ *M* to minimize cytotoxicity. Based on these results, glyphosate did not appear to affect hormone binding at the hERα, hERβ, or hAR.

In Walsh et al. (2000), investigators assessed whether glyphosate or Roundup might 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). It was postulated 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 may potentially produce adverse effects. In this study, Roundup (180 g/L glyphosate) significantly inhibited steroidogenesis (as seen by decreased progesterone production in MA-10 cells) by inhibiting StAR protein expression at concentrations of 20–100 μg/ml. It is noteworthy that glyphosate alone, however, did not exert an 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 2 h 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 nonstimulated MA-10 cells. Exposure to the surfactants, Roundup-branded formulation, and Roundup blank resulted in reduced levels of the 30-kD form of StAR protein, but not the 37-kD precursor form. Because formation of the 30-kD form requires mitochondrial import and processing of the 37-kD precursor, the effect of treatment on mitochondrial potential, an indicator of proper mitochondrial membrane function, was measured using the JC-1 cationic dye. Treated MA-10 cells demonstrated a loss of normal mitochondrial membrane potential, implying that proper import and processing of the 37-kD form of the StAR protein was disrupted upon treatment. This finding explains the previously observed decrease in the 30-kD form of the StAR protein. In addition, this effect on mitochondrial membrane potential was seen 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 support the concept that the adverse effects of Roundup branded herbicidal formulations on steroidogenesis are not mediated by glyphosate exposure, but rather are due to a nonendocrine mechanism of compromised mitochondrial membrane potential and altered permeability of cell membranes.

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 P-450 activity of plants (Lamb et al. 1998), it was postulated that mammalian aromatase (also a cytochrome P-450 enzyme) might be adversely affected. It was also of interest to further investigate claims made in other studies that glyphosate and/or an unspecified Roundup branded formulation induced reproductive/developmental disturbances. The "Roundup" formulation was diluted in water to concentrations of $\leq$2% based on the recommended concentration for agricultural use of 1–2% in water. Concentrations of pure glyphosate equivalent to those present in the range of Roundup dilutions tested were also used. Aromatase activity was measured at 1 and 18 h post treatment by determining the amount of tritiated water released from the radiolabeled aromatase substrate, [1β^{-3}H]-androstenedione. RT-PCR to amplify aromatase and GAPDH (as an endogenous control) mRNA was performed. General cell viability was also measured. Roundup exerted a more pronounced effect on cell viability than equivalent concentrations of pure glyphosate, indicating that the formulation ingredients played an important role in cytotoxicity, as discussed previously for in vitro systems where surfactants were added. Pure glyphosate did not significantly affect aromatase activity at 1 or 18 h at any concentration tested ($\leq$0.8%, or the highest concentration at which marked cytotoxicity was not observed). Similarly, aromatase mRNA levels were not affected by 18 h of treatment with $\leq$0.1% glyphosate. Incubation of the cells in Roundup for 1 h, however, increased aromatase activity at all concentrations examined (0.02–0.2%). In contrast, incubation in Roundup for 18 h induced a concentration-dependent decrease in aromatase activity at all concentrations tested ($\leq$0.8%). Levels of aromatase mRNA were also significantly decreased upon 18 h of incubation with 0.02 and 0.06% concentrations of Roundup. It was noted that

if glyphosate was combined with 0.02% Roundup, a greater fall in aromatase activity after 18 h of incubation was observed than was seen with 0.02% Roundup alone; however, the concentration of pure glyphosate used in this experiment was not indicated. Richard et al. (2005) also measured aromatase activity in microsomes prepared from human full-term placental tissues incubated for 15 min 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 min post treatment, the fall in aromatase activity likely is not due to cell death. Based on additional experiments using microsomes derived from equine testis, it was concluded that the rapid decrease in microsomal aromatase activity is due to competitive inhibition; however, only data using Roundup are presented in the study. Based on these results, Richard et al. (2005) concluded that the additives in Roundup play a key role in its effect on aromatase, but that glyphosate itself might also elicit adverse effects. Although it was shown that pure glyphosate added to Roundup further reduced 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 needs to 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 nonvalidated in vitro system using physiologically irrelevant concentrations and Richard et al. (2005) were thought to have greatly overinterpreted the results of their studies, the French Ministry of Agriculture and Fish concluded that the study provided no useful information that was of value 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.

A similar study using both JEG3 cells and the human embryonic kidney 293 cell line was conducted in the same laboratory to assess the effects of 1–2% concentrations of Roundup Bioforce (360 g/L acid glyphosate) and equivalent glyphosate concentrations on cell viability and aromatase activity (Benachour et al. 2007). The glyphosate solution used in many of these experiments was reported to have been pH adjusted to 5.8 (equivalent to the pH of 2% Roundup Bioforce solution). Following 1, 24, or 48 h of incubation, 293 cells were found to be more sensitive to the cytotoxic effects of treatment than JEG3 cells; cells in serum-free media were more sensitive than those incubated in serum-containing media; and Roundup Bioforce was shown to be substantially more cytotoxic than glyphosate itself. In additional experiments, both Roundup Bioforce and glyphosate reduced aromatase activity in 293 cells cultured for 24 h in serum-free medium and human placental microsomes treated for 15 min. Roundup Bioforce was also demonstrated to affect aromatase activity in equine testicular microsomes, and this effect appeared to be temperature responsive. The sensitivity of the cells incubated in serum-free media is not surprising. Serum supplementation of culture media provides cells with necessary nutrients and other protective elements. Along these lines, it was reported that cells grown in the absence of serum were not viable after 60 h, regardless of treatment. Benachour et al. (2007) interpreted these results to suggest that glyphosate is cytotoxic and possesses endocrine-disrupting properties. Because many of these experiments were conducted using serum-free media and the pH of the glyphosate solution was only adjusted to be equivalent to that of Roundup and not physiological pH, however, it is likely that many of the effects observed following treatment are due to changes in pH rather than a direct effect of glyphosate on cells. Ideally, the pH of the glyphosate solution should have been adjusted to physiological pH for these experiments. Alternatively, a negative control treatment using media that was pH adjusted to 5.8 should have been included. Interestingly,

in at least one of the experiments measuring the effects of Roundup treatment on aromatase activity in microsomal preparations, the pH of the Roundup was adjusted to physiological pH (7.4). Why the pH of the glyphosate solution was not similarly adjusted in these experiments is not clear. Given the confounding surfactant effects of damaging cell membranes, the value of these data is questionable.

Follow-up investigations were conducted by Benachour and Séralini (2009) using endothelial cells from human umbilical cord vein (HUVEC), 293 embryonic kidney cells, and JEG3 placental cells. These studies assessed the cytotoxic potential and apoptosis associated with glyphosate, four glyphosate-based formulations, AMPA, and POEA. Thus, endocrine activity was not specifically evaluated and these studies are not discussed in detail herein.

Gasnier et al. (2009) assessed the potential for endocrine disruption in HepG2 cells. Additional experiments reported in this study evaluated cytotoxicity and genotoxicity, but these results are beyond the scope of the present analysis and are not discussed herein. In these experiments, both glyphosate and various formulations used were pH 5.8 prior to cell treatment. To evaluate effects on aromatase activity, HepG2 cells in serum-free media were exposed for 24 h to non-cytotoxic concentrations of either glyphosate or one of four different commercial glyphosate-based formulations (Roundup Express with 7.2 g/L glyphosate; Bioforce/Extra 360 with 360 g/L glyphosate; Grand Travaux with 400 g/L glyphosate; or Grand Travaux plus with 450 g/L glyphosate). After treatment, cells were washed, and then treated with 200 n*M* of radiolabeled androstenedione. Aromatase mRNA levels were also measured by semiquantitative RT-PCR. Experiments were repeated thrice in triplicate. Glyphosate alone at concentrations of up to 0.3% exerted no marked effect on aromatase activity or mRNA levels. In contrast, the four herbicide formulations—all of which contain various surfactants—inhibited aromatase activity and altered aromatase mRNA levels. Gasnier et al. (2009) noted that these effects were not proportional to the amount of glyphosate in the formulation, which suggests that the findings were due to other formulation components. In further experiments, HepG2 cells that had been transiently transfected with human ERα, ERβ, and a luciferase-linked estrogen-responsive reporter gene were treated in serum-free media with either glyphosate or 1 of the 4 formulations for 24 h. These incubations were done in the presence of 10 n*M* 17β-estradiol to assess antiestrogenic potential. To assess antiandrogenic potential, MDA-MB-453-kb2 cells, which contain endogenous androgen receptors and a stably transfected androgen-responsive reporter gene, were treated in serum-free media with either glyphosate or 1 of the 4 formulations for 24 h in the presence of 0.4 n*M* dihydrotestosterone. Glyphosate alone exerted no significant antiestrogenic activity. At low concentrations, glyphosate treatment exhibited some antiandrogenic activity; however, as the glyphosate concentration increased, androgenic activity returned. Because the antiandrogenic activity did not increase with increasing glyphosate concentrations, it is unlikely to be related to treatment. In contrast, the four herbicide formulations inhibited both estrogenic and androgenic activity. Again, these findings were not proportional to the amount of glyphosate in the formulations, suggesting the effects were due to the presence of other components in the formulations. As a whole, the results of this study suggest that glyphosate did not markedly affect endocrine activity. Further, as with other studies from this same group of investigators (Benachour et al. 2007; Benachour and Séralini 2009; Richard et al. 2005), this study is confounded by the use of commercial formulations containing surfactants and other components that affect the integrity of cellular membranes and consequently produce false findings.

Hokanson et al. (2007) examined gene expression in MCF-7 cells in response to treatment with 0.0001–0.1% dilutions of a herbicidal formulation containing 15% glyphosate (exact formulation not specified). Following 18 h of exposure, the expression of 1550 genes in treated and control cultures was evaluated

using a DNA microarray platform. Data showed that 680 genes were either upregulated or downregulated in response to glyphosate treatment; however, it is not clear whether the variability in gene expression of control cells was taken into account. The expression of seven of the genes was then examined in more detail using quantitative PCR. In this analysis, only three of the seven genes evaluated continued to display up- or downregulation; the other four failed to show disregulation in response to treatment. The 3 genes that continued to demonstrate a treatment-related effect (hypoxia inducible factor 1 [HIF1], early growth response 1 [EGR1], and chemokine ligand 12 [CXCL12]) were said to also be affected by treatment with 3×10^{-10} *M* estrogen, which induced a response that was intermediate between that of control and treatment with estrogen plus herbicide.

Hokanson et al. (2007) interpreted these results to mean that glyphosate treatment altered estrogen regulation of gene expression; however, it cannot be determined whether the gene response may be due to formulation ingredients besides glyphosate or an effect of treatment on pH of the cell culture media. Furthermore, no evidence exists in the study to suggest that the effect of herbicide treatment was mediated through an estrogen-related pathway.

Paganelli et al. (2010) studied the potential for glyphosate or a glyphosate-based herbicide formulation (Roundup Classic, with 48% w/v glyphosate salt) to induce malformations in developing *Xenopus laevis* embryos. Embryos at the 2-cell stage were exposed to 1/3000, 1/4000, or 1/5000 dilutions of Roundup Classic for an undisclosed period of time. Compared to untreated controls, treated embryos exhibited downregulation of the neural crest markers *slug, krox-20* in rhombomere 3, and *N-tubulin* along the three longitudinal domains of the posterior neural plate. In addition, the expression of *shh* (a morphogen), *pax6* (essential for eye formation), *otx2* (gene expressed in various parts of the developing eye), and *sox9* (a transcription factor expressed in cranial neural crest cells) was reduced in the embryos with herbicide formulation treatment at a 1/5000 dilution. In further experiments, embryos were directly injected with 500 pg glyphosate, again for an undisclosed period of time. Similar, albeit milder, downregulation of these neural crest markers was observed on the injected side of the embryos. To assess the functional changes associated with these changes in neural crest marker expression, embryos treated at the 2-cell stage were allowed to develop to stage 47, and then stained for skeletal analysis. Those treated with the herbicide formulation exhibited reduced cranial structures and eyes. Glyphosate injection resulted in similar malformations. Additional experiments using embryos transiently transfected with a retinoic acid-responsive reporter gene suggested that treatment with the herbicide formulation enhanced endogenous retinoid activity and that this increased activity played a role in the induction of cranial malformations. Finally, chick embryos were treated in culture with the herbicide formulation and showed a similar downregulation of neural crest markers as was observed in the *Xenopus* embryos. The significance of these findings is unclear for several reasons. One drawback relates to the fact that glyphosate was not reported to have been pH adjusted; thus, the reported changes may have been due to the acidic nature of the test compound. Further, injection is an inappropriate route of exposure for assessing risk and it is not clear why the glyphosate was injected into the embryos rather than administered in the culture media like Roundup Classic. Overall, these findings require further substantiation in other labs using appropriate methods before the observations can be considered for risk assessment.

Summary—Endocrine Disruption Overall, these studies do not suggest that glyphosate is an endocrine disruptor. When tested alone, glyphosate was shown to be not estrogenic in a number of assay systems. Glyphosate did not activate the estrogen receptor or affect its ability to bind its normal endogenous ligand in either in vitro or in vivo test systems (Gasnier et al. 2009; Kojima et al. 2004;

Petit et al. 1997; Xie et al. 2005); glyphosate also failed to displace estradiol from human sex hormone-binding globulin (Meulenberg 2002). Although a Roundup-branded formulation was able to alter StAR protein function (Walsh et al. 2000) and aromatase activity (Richard et al. 2005; Benachour et al. 2007), and inhibit progesterone production (Levine et al. 2007), these same effects generally were not observed when glyphosate was tested alone, suggesting that the responses might be due to another component of the pesticide formulation—likely a surfactant, as shown in the study by Levine et al. (2007), and likely via a non-endocrine-mediated mechanism. Finally, while both Roundup and its active ingredient, glyphosate, were able to induce the proliferation of estrogen-responsive MCF-7 cells in culture (Lin and Garry 2000), the use of steroid growth factor-deficient serum suggested that this response was not mediated through an estrogenic pathway.

Reproductive Function

Yousef et al. (1996) investigated the impact of glyphosate, as well as that of other pesticides, on the motility of human and rabbit sperm in vitro. This study was done, in part, to evaluate the utility of the motile rabbit spermatozoa assay as a test system for predicting human responses to male reproductive toxicants. The concentration of glyphosate used cannot be determined because the study indicates that a glyphosate-based herbicide, and not pure glyphosate, was used in these experiments, and neither the commercial name nor the glyphosate concentration of this formulation was provided. Following incubation of sperm with varying concentrations of pesticides in either protein-free medium or medium containing bovine serum albumin (BSA), a sperm motility index (SMI) was calculated. This index was based on the percentage of sperm that were motile and the motility grade of the sperm (with values ranging from 0 in cases of no motility to 4 for cases of fast forward progressive movement). Fifteen minutes of incubation in BSA medium containing what was reported as 250, 500, or 1000 μM of the glyphosate-based test solution resulted in rabbit SMI values of 2.4, 2, and 1.8, respectively, versus a control SMI of 3.5. In contrast, the glyphosate-based test solution administered in protein-free medium for 15 min resulted in a rabbit SMI value of 0, regardless of the concentration, versus a control SMI value of 2.7. Following 60-min incubations with varying concentrations of the glyphosate-based test solution, the IC_{50} values for rabbit sperm were 23.3 μM and 500 μM in protein-free medium and BSA medium, respectively. Similarly, the IC_{50} values for human sperm motility were 48.2 μM and 740 μM in protein-free medium and protein-containing (BSA) medium, respectively. Although these results suggested that the protein present in BSA-containing medium partially protected sperm from the harmful effects of treatment, little else can be concluded from this study. Because a herbicidal formulation was used rather than pure glyphosate, it is consistent with the aforementioned and reviewed studies that the observed results were due to the presence of surfactant rather than glyphosate. Furthermore, Yousef et al. (1996) did not mention whether they corrected the pH of the media following the addition of the pesticides. Certainly, a pH outside the normal range would adversely impact sperm motility, regardless of treatment agent. Thus, the observed effects may have little to do with the actual agent administered in the study. Overall, this study provides no useful information regarding the potential adverse reproductive effects of glyphosate for men.

Conclusions—Mechanistic Studies

Overall, the aggregate of available mechanistic data did not provide a plausible MOA by which glyphosate may produce adverse developmental or reproductive effects in humans. Many of these studies provide inadequate description of the test agent(s)—particularly, whether test systems were treated with pure glyphosate or a glyphosate-based commercial herbicide—and the final concentrations of glyphosate to which test models were exposed. These deficiencies make it impossible to determine whether the observed

results may be attributed to glyphosate or another formulation ingredient, such as the surfactant. Furthermore, in the only study to test for this possibility (Levine et al. 2007), the results demonstrated that the observed effects were mediated through the surfactants present in the herbicidal formulations and consumer products. Finally, for the purposes of a human health risk assessment, these data provide little relevant information. For one, the concentrations administered in these in vitro studies are substantially higher than those anticipated to be experienced as a result of dermal contact or oral ingestion of glyphosate. In addition, these studies, by their very nature, do not take into account such factors as absorption, distribution, metabolism, and elimination, all of which play important roles in shaping human exposure responses. In conclusion, these data do not show a plausible and consistent mechanism by which glyphosate might produce developmental or reproductive disturbances in humans or animals.

EVALUATION OF BIOMONITORING DATA

Although the preceding hazard assessment for glyphosate failed to demonstrate any consistent evidence to indicate that glyphosate exposure may produce adverse developmental or reproductive health effects in humans, a review of the available biomonitoring data was considered pertinent to this evaluation in order to better understand the reasonably anticipated exposure levels for humans.

Biomonitoring Studies

To date, only a small body of biomonitoring data exists for assessing exposure levels associated with glyphosate field application (Table 12). These data are derived from studies looking at occupational pesticide levels in tree nursery workers (Lavy et al. 1992; 1993), those involved in the spray-clearing of brush (Cowell and Steinmetz 1990a; Jauhiainen et al. 1991), and members of farm and nonfarm families (Acquavella et al. 2004; 2005; Baker et al. 2005; Curwin et al. 2007a; 2007b; Mandel et al. 2005). Two other biomonitoring studies of glyphosate have been published (Abdelghani 1995; Centre de Toxicology du Quebec 1988), but neither study provides measures of individual systemic glyphosate concentrations, and thus they are not discussed in this review. Studies that measured glyphosate exposures via passive dosimetry only (for example, on clothing, in air samples, or through hand washes alone) were also excluded from analysis, as these types of exposure measures do not provide a predictive indicator of internal dose.

TABLE 12. Estimated Glyphosate Doses Associated With Herbicide Application

Study	Sample size	Dosimetry method	Estimated glyphosate dose	LLOMV[a]
Spray-clearing of brush				
Cowell and Steinmetz 1990	16	Urinalysis (5/16 participants)	18.8 μg	0.01 μg/ml
		Passive (patch)	274 μg	0.1 μg/patch
Jauhiainen et al. 1991	5	Urinalysis	ND[b]	0.1 μg/ml[c]
		Passive (air)	≤15.7 $\mu g/m^3$	0.3 $\mu g/m^3$[c]
Tree nursery work				
Lavy et al. 1992; 1993	14	Urinalysis	ND	0.01 μg/ml
Farm and nonfarm families				
Acquavella et al. 2004	48 farmers	Urinalysis	4 μg/kg[d]	0.001 μg/ml[c]
	48 spouses		0.04 μg/kg[d]	
	78 children		0.8 μg/kg[d]	
Curwin et al. 2007a; 2007b	65 farm children	Urinalysis	0.11 μg/kg[e]	0.0009 μg/ml[c]
	51 nonfarm children		0.13 μg/kg[e]	

Note. 1 ppb = 1 μg/L = 1 μg/1000 ml = 0.001 μg/ml. [a]LLOMV = Lower limit of method validation. [b]ND = Not detectable. [c]Assay detection limit. [d]Based on highest reading registered. [e]Based on maximum likelihood model.

Cowell and Steinmetz (1990a) measured glyphosate concentrations in the urine of forestry workers involved in the mixing and backpack spray application of a Roundup herbicide at three different locations. Although all 16 workers were involved in spray application of the herbicide, only 1 worker at each site prepared and mixed the Roundup herbicide prior to application. Air samples from the breathing zone of each worker were collected using an air filter and portable pump. Passive monitoring was conducted using hand washes and gauze patches placed at various predetermined locations on the workers' clothing. To determine the percent clothing penetration, patches were also worn underneath the clothes at sites adjacent to those where outside patches were attached. Urine samples were collected on the day before, the day of, and 3 d following herbicide application. Twelve-hour composite samples from each worker were analyzed. Following sample processing, glyphosate was quantified using high-pressure liquid chromatography and fluorescence detection. The lower limit of method validation (LLOMV) was reported to be 0.01 μg/ml for the urine samples, 0.5 μg for each air filter, and 0.1 μg per patch. For the purposes of exposure assessment, data less than the LLOMV were assumed to be equal to one-half the LLOMV. Applicator body doses were calculated based on the first 72 h following application. Only 5 of 16 workers had measurable glyphosate concentrations in their urine on the day of application; all other urine samples were below the limits of detection. Based on analysis of the collected urine samples, the estimated average total body dose following spray application was 18.8 μg. In comparison, the estimated average total body dose based on passive dosimetry measures was 274 μg and the average inhalation dose based on air sampling was 55.3 μg. Total body dose did not appear to correlate with specific occupation (mixing versus spray application). These data show that passive dosimetry estimates are approximately one order of magnitude higher than those based on biological measures.

Jauhiainen et al. (1991) measured glyphosate concentrations in air and urine samples from five workers employed in the spray-clearing of forest brush. Workers were involved in the daily mixing of their own herbicide sprays, wore limited protective equipment (primarily helmets and gloves), and did not have access to wash facilities during their workday. A control group of five forest workers involved in the planting of trees was also evaluated. Air samples from the breathing zone of the workers were taken daily for 1 wk using a portable pump. Sampling times varied from 1 to 6 h. Urine samples were collected over the test week at the end of each workday, as well as after a 3-wk follow-up period. Following sample processing, glyphosate concentrations were measured by gas chromatography, with a detection limit of 0.1 ng/μl (0.3 $\mu g/m^3$). Mid-week air samples contained less than 1.25 μg glyphosate/m^3 air. The highest recorded air sample readings were 2.8 and 15.7 $\mu g/m^3$. All urine glyphosate concentrations were below the limits of detection.

Lavy et al. (1992; 1993) measured glyphosate exposure levels among conifer seedling nursery workers. Fourteen workers—including applicators, weeders, and scouts—were employed at two tree nurseries that used a Roundup herbicide. In this study, three different types of measurements were taken to assess potential and real exposures: dislodgeable residues, passive monitoring, and biological monitoring. To assess the amount of residual glyphosate that could be dislodged from conifer seedlings during contact with the plants, 100-g samples of fresh seedlings were shaken and rinsed under water for 45 s each. These measurements were made twice weekly over four spring/summer months. Passive monitoring of exposures was conducted using gauze patches attached to the clothing of workers at nine potential exposure points and via hand rinses of the workers taken at the end of the same workday. These measurements were taken 1 d/wk over the entire course of study and composited for each day of measurement to provide total passive exposures for each

worker. Biological monitoring involved collection of total daily urine for each worker over 12 consecutive weeks. Twenty-four-hour samples were also collected once weekly for 5 mo following the study period for each worker. Glyphosate concentrations were determined using the analytical procedures of Cowell and Steinmetz (1990b). The limit of detection for urine samples was 0.002 ppm and the lower limit of method validation was defined as 0.01 ppm. Of the 78 dislodgeable residue samples taken at 21 different sampling times, only 1 sample was positive for glyphosate residue, measuring 138.5 μg glyphosate. This finding indicates that dislodgeable residues are not a significant source of glyphosate exposure for nursery workers. Passive exposure measurements indicated that ankles and thighs received the greatest exposure, with 98% of exposures occurring at or below the thigh. Applicators received greater exposures than weeders. Scouts showed minimal exposure, with only 1 of 23 hand washes and 1 of 34 composited patch samples being positive for glyphosate. Normalizing the composite exposure values for body weight and exposure period resulted in average exposure levels of 7.2×10^{-4}, 2.0×10^{-4}, and 1.6×10^{-6} mg/kg/h for applicators, weeders, and scouts, respectively. In total, 355 urine samples were analyzed from the 14 workers over the course of study; however, all samples were below the limits of detection for glyphosate. These results suggested that, despite the level of passive exposures measured, actual internal doses of glyphosate received by the workers were minimal to nonexistent.

The Farm Family Exposure Study was initiated in 1999 and ultimately involved the biomonitoring of 95 families for glyphosate, 2,4-D, and chlorpyrifos exposure during years 2000 and 2001 (Acquavella et al. 2004; 2005; Baker et al. 2005; Mandel et al. 2005). Only the results related to glyphosate application are discussed herein. Families were randomly selected from listings of licensed pesticide applicators in South Carolina and Minnesota. Eligibility requirements were as follows: The family had to consist of the farmer, spouse, and at least 1 child between the ages of 4 and 18 yr; the family had to live on the farm and to farm at least 10 acres within 1 mile of the home, onto which it planned to apply 1 or more of the study pesticides within the study period as a part of normal operations; and the family members had to be willing to collect 24-h urine samples over 5 d, starting 1 d prior to the pesticide application through 3 d following application. Parents filled out pre- and postapplication questionnaires detailing family activities and application practices. In addition, trained field staff were on hand to observe the pesticide application. Forty-eight of the 95 families provided specimens related to glyphosate application; these included specimens for 79 children. Urine samples were analyzed for glyphosate using chelation ion exchange to concentrate and isolate the pesticide, followed by high-pressure liquid chromatography and fluorescence detection. Glyphosate findings were adjusted for recovery of the analyte using values obtained from spiked field- and travel-samples. Recovery was 69% for a 10-ppb sample and 78% for 100-ppb samples. The detection limit was 1 μg/l for a 100-ml urine sample.

Twenty-nine percent of farmers applied glyphosate within 1 wk prior to their participation in the Farm Family Exposure Study. Glyphosate was applied using a tractor and boom sprayer in all cases. Twenty-nine percent of these farmers did not wear rubber gloves during the application process, 15% spilled pesticide during the mixing and/or loading stages of application, and 27% worked on their equipment during the application process. Only 60% of farmers had detectable glyphosate levels in their urine on application day, the day of highest glyphosate readings. By 3 d postapplication, this number had declined to 27%. Urine concentrations of glyphosate ranged from below the limit of detection to 233 ppb. The geometric mean value for farmers was 3.2 ppb on application day, and declined to 1 ppb by postapplication day 3. Use of rubber gloves exerted the greatest influence on urinary concentrations. Other factors associated with urine concentrations of glyphosate in

the farmers included the number of times the farmers mixed and loaded the glyphosate, use of an open-cab tractor, observed skin contact with the pesticide, and repair of the application equipment. The number of acres treated exerted no significant influence on urinary glyphosate concentrations.

Only 2 of 48 spouses had detectable glyphosate concentrations in their urine on application day. The highest urine concentration of glyphosate in a spouse was 3 ppb. No spouses participated in the pesticide application process. Nine of 78 children had detectable glyphosate concentrations in their urine on the day of application; all but 1 of these were reported either to have been present during or to have helped with the pesticide application. The highest glyphosate urinary value in a child was 29 ppb.

Systemic doses of glyphosate were calculated for all participants with detectable urine glyphosate concentrations. For each individual, the total amount of glyphosate excreted during the study period was determined, adjusting for incomplete excretion and pharmacokinetic recovery; this value was then divided by each individual's body weight for determination of an individual's systemic dose. Using these calculations, the maximum systemic dose for farmers was estimated to be 0.004 mg/kg and the geometric mean value was estimated to be 0.0001 mg/kg. Maximal systemic doses for spouses and children were estimated to be 0.00004 mg/kg and 0.0008 mg/kg, respectively. These values are all well below the oral reference dose for glyphosate of 2 mg/kg/d set by the U.S. Environmental Protection Agency (U.S. EPA 1993).

Curwin et al. (2007a; 2007b) conducted a similar study of both farm and nonfarm families residing in Iowa during the spring and summer of 2001. Exposure to seven target pesticides (atrazine, acetochlor, metolachlor, alachlor, chlorpyrifos, glyphosate, and 2,4-D) was examined; however, only the results for glyphosate are discussed herein. Study recruitment was done by convenience sampling. Study eligibility requirements were as follows: Households had to reside in 1 of 10 counties in central or eastern Iowa and have at least 1 child under the age of 16 yr; nonfarm families had to reside on land that was not used for farming and no one in the household could be employed in agriculture or the commercial application of pesticides; farm families had to use at least 1 of the 7 target pesticides. Twenty-five farm families (66 farm children) and 25 nonfarm families (52 nonfarm children) were enrolled in the study. Each household was visited twice during the study period and two urine samples were collected from participants at each visit (one from the evening and one from the following morning). Dust samples were collected during each visit according to standard practices established by the American Society for Testing Material (ASTM). Urine samples were kept cool, then shipped frozen to the laboratory, where the samples were analyzed for parent pesticides and metabolites by immunoassay. The limit of detection (LOD) for glyphosate was 0.9 μg/L. Urinary concentrations data were recorded as positive values at or above the LOD, positive values below the LOD, or nondetects. These data were then analyzed using two different approaches. In the maximum likelihood estimation, urinary concentrations reported as either nondetects or at levels below the LOD were set at the LOD for the assay. In the mixed-effects modeling approach, positive urinary concentrations below the LOD were used as reported and nondetects were set at one-half the lowest positive concentration measured. Urinary creatinine levels were also measured and used to normalize for total daily urinary voids when estimating daily pesticide exposures. Only 30% of absorbed glyphosate was assumed to be excreted in the urine, and this information was used to correct for total glyphosate exposure.

In the case of glyphosate, urinary concentrations were above the limits of detection for 65–75% of the parent samples and for 81–88% of children's samples. Furthermore, farm and nonfarm families did not significantly differ in their mean urinary concentrations of glyphosate. Curwin et al. (2007a; 2007b) surmised that this may be because glyphosate use is not restricted to agricultural practices,

but rather may be commonly seen in residential settings as well. Geometric mean urinary concentrations of glyphosate (using the maximum likelihood model) were 1.4 μg/L (range: 0.13–5.4 μg/L) and 1.9 μg/L (range: 0.020–18 μg/L) in nonfarm and farm fathers, respectively; 1.2 μg/L (range: 0.062–5 μg/L) and 1.5 μg/L (range: 0.1–11 μg/L) in nonfarm and farm mothers, respectively; and 2.7 μg/L (range: 0.1–9.4 μg/L) and 2 μg/L (range: 0.022–18 μg/L) in nonfarm and farm children, respectively. Mean urinary concentrations calculated using the mixed-effect model were similar. These estimated urinary concentrations of glyphosate from this study are all within the same approximate order of magnitude as those found in the Farm Family Health Study, discussed earlier.

Based on these data, the geometric mean doses of glyphosate were estimated for both farm and nonfarm children. Again using the maximum likelihood model, the daily absorbed dose of glyphosate for farm children was estimated to be 0.11 μg/kg/d (range: 0.013–0.34 μg/kg/d). This was similar to the dose estimated for nonfarm children: 0.13 μg/kg/d (range: 0.037–0.33 μg/kg/d). However, these values are approximately eightfold lower than the 0.8-μg/kg/d glyphosate exposure estimated for farm children in the Farm Family Exposure Study and certainly lower than the oral reference dose for glyphosate of 2 mg/kg/d set by the U.S. EPA (U.S. EPA 1993). The reason for the discrepancy in values between the two studies is not clear, but likely relates to differences in adjustments made to account for total urinary void and incomplete excretion of glyphosate.

Summary—Biomonitoring Data

The body of biomonitoring data available for glyphosate is limited at this time. Nevertheless, the data reviewed herein clearly show that the degree of systemic glyphosate exposure that occurs as a result of normal application practices is exceedingly small, often below the limits of detection (especially for those not intimately involved in the application process). In fact, the highest systemic dose estimated from these studies was 0.004 mg/kg (Acquavella et al. 2004), a value 500-fold below the daily oral reference dose for glyphosate of 2 mg/kg/d (U.S. EPA 1993). These findings indicate that the risk of substantial exposure as a result of glyphosate application practices is minimal at best.

CONCLUSIONS

An extensive, in-depth analysis of the available scientific literature provides no apparent evidence to indicate that exposure to glyphosate is associated with the potential to produce adverse developmental and reproductive effects in humans. While the body of epidemiological data for glyphosate is fairly limited, and none of the available studies (with the exception of Sanin et al. 2009) were designed specifically to assess the potential effects of glyphosate exposure, data as a whole reveal no developmental or reproductive health disturbances associated with exposure. In contrast to epidemiological data, the database of animal studies for glyphosate is relatively robust, including studies of mice, rats, and rabbits exposed to glyphosate, various glyphosate-based herbicidal formulations, the major glyphosate environmental breakdown product AMPA, and POEA surfactants included in some Roundup-branded herbicides. All guideline-compliant studies reviewed found no marked effects of glyphosate treatment on reproductive health or the developing offspring at non-maternally toxic doses (Holson 1990; 1991; IRDC 1980a; 1980b; Knapp 2007; 2008; Reyna 1990;Schroeder 1981). It should be noted that while a number of non-guideline-compliant studies claimed adverse developmental effects associated with glyphosate exposure (Beuret et al. 2004; Dallegrave et al. 2003; Dariuch et al. 2001; Yousef et al. 1995), these investigations suffer from numerous inadequacies in design, which makes substantiation of their conclusions problematical. Furthermore, these studies all used commercially formulated glyphosate-based herbicides rather than pure

glyphosate. Thus, findings reported in these studies cannot be definitively assigned to glyphosate exposure.

Similarly, review of the available mechanistic data related to glyphosate fails to find a plausible MOA by which glyphosate may be able to induce adverse developmental or reproductive outcomes. It should be noted, however, that the body of available studies suffers from numerous design inadequacies, particularly with regard to the type of test agents used (commercially available glyphosate-based herbicides versus pure glyphosate). Furthermore, other than hypothesizing possible MOA, these data provide little relevant information that can be used in a human health risk assessment.

Finally, a review of the limited body of available biomonitoring studies shows that, via reasonably anticipated exposure routes, human exposure to glyphosate is likely to be well below the daily oral reference dose for glyphosate of 2 mg/kg/d, as set by the U.S. EPA (1993). These data show that, regardless of any potential developmental and reproductive hazards that may be alleged based on misinterpretation of results from animal and mechanistic studies, the levels of glyphosate to which humans are likely to be exposed are far below the range of doses considered to be safe by the U.S. and other regulatory agencies worldwide. In conclusion, a thorough evaluation of the available data demonstrates that exposure to environmentally relevant glyphosate concentrations is not anticipated to produce adverse developmental and reproductive effects in humans.

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