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Effects of Glyphosate and its Formulation, Roundup, on Reproduction in Zebrafish (*Danio rerio*)

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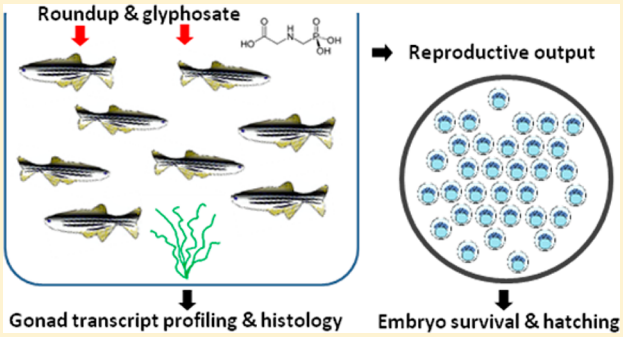
Supporting Information

ABSTRACT: Roundup and its active ingredient glyphosate are among the most widely used herbicides worldwide and may contaminate surface waters. Research suggests both Roundup and glyphosate induce oxidative stress in fish and may also cause reproductive toxicity in mammalian systems. We aimed to investigate the reproductive effects of Roundup and glyphosate in fish and the potential associated mechanisms of toxicity. To do this, we conducted a 21-day exposure of breeding zebrafish (*Danio rerio*) to 0.01, 0.5, and 10 mg/L (glyphosate acid equivalent) Roundup and 10 mg/L glyphosate. 10 mg/L glyphosate reduced egg production but not fertilization rate in breeding colonies. Both 10 mg/L Roundup and glyphosate increased early stage embryo mortalities and premature hatching. However, exposure during embryogenesis alone did not increase embryo mortality, suggesting that this effect was caused primarily by exposure during gametogenesis. Transcript profiling of the gonads revealed 10 mg/L Roundup and glyphosate induced changes in the expression of *cyp19a1* and *esr1* in the ovary and *hsd3b2*, *cat*, and *sod1* in the testis. Our results demonstrate that these chemicals cause reproductive toxicity in zebrafish, although only at high concentrations unlikely to occur in the environment, and likely mechanisms of toxicity include disruption of the steroidogenic biosynthesis pathway and oxidative stress.



INTRODUCTION

Glyphosate is extensively used worldwide, topping lists of agricultural herbicide usage in Europe[1] and the U.S.[2] It is a broad-spectrum, post emergence herbicide, which acts by binding phosphoenolpyruvate, the substrate of EPSP synthase, and subsequently inhibiting aromatic amino acid synthesis via the shikimate pathway in plants.[3,4] Glyphosate is generally applied as part of a formulated product, the most widely used of which are the Roundup herbicides. Roundup formulations contain glyphosate in the form of an isoproylamine salt, which aids solubility but does not affect its properties as the active ingredient, together with various adjuvants which enhance its herbicidal properties. One of the most important and commonly used adjuvants is polyethoxylated tallow amine (POEA), a surfactant that enhances penetration of glyphosate through the plant cuticle.[5,6] Glyphosate and Roundup are also extensively used as domestic and urban-area weed-killers.[2] Commercial glyphosate formulations vary in composition with country and purpose and the properties of these formulations, including their toxicity, can be compared using the concentration of glyphosate present, expressed as glyphosate acid equivalent (a.e.).

Glyphosate is known to strongly adsorp to soil, where it is subject to microbial degradation. This is one of glyphosate's advantageous herbicidal properties, limiting agricultural input to surface waters in ideal conditions. However, pulses of contamination can be expected when rainfall occurs directly after application and when flood events increase river sediment load.[6] Urban runoff and wastewater treatment effluent also account for considerable glyphosate input into rivers.[7] Despite its widespread use, concentrations of glyphosate, or its associated formulation components, are not routinely monitored in surface waters. However, glyphosate concentrations worldwide have been regularly reported to occur up to ~10−15 μg/L in rivers [e.g., refs 8 and 9]. Considerably higher peaks in concentration, in the high micrograms per liter range, have also been measured but are mainly associated with direct aquatic application and in isolated wetland environments.[6,10]

Although the target mechanism of action of glyphosate and glyphosate-based formulations is specific to plants, they have been shown to induce diverse biological effects in a range of nontarget organisms. In fish, much of previous research assessing effects of Roundup and glyphosate has focused on their induction of oxidative stress through ROS generation or interference with cellular antioxidant production. Short-term exposures (up to 6 days) to 1−20 mg/L of several Roundup formulations in a number of fish species altered levels of cellular

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antioxidants and induced oxidative damage of DNA, lipids, and proteins [e.g., refs 11−15]. Environmentally relevant concentrations of Roundup, glyphosate and POEA have also induced DNA damage in blood and liver cells of eel and catfish after up to 9 days exposure.[16−19] Other studies have found that Roundup, and in some cases glyphosate, induce other effects in fish including neurotoxicity and immunotoxicity [e.g., refs 20−22]. Similar evidence of Roundup and glyphosate toxicity has been found for other vertebrate species, and demonstrated effects include occurrence of developmental abnormalities, especially in amphibians.[23,24] Roundup formulations have largely been found to be more toxic than pure glyphosate. The inherent toxicity of POEA,[17,23] and potentially other formulation components, is likely to contribute to this, although a modulating effect on glyphosate toxicity is also possible. Few studies, however, have directly compared equivalent concentrations of Roundup and glyphosate.

The potential of Roundup to disrupt the endocrine system *in vitro* has been demonstrated in mammalian cell lines. In mouse Leydig cells, sublethal concentrations of Roundup (from 25 mg/L) altered the transcription and activity of steroidogenic acute regulatory protein (StAR), resulting in disruption of progesterone production.[25] A number of studies demonstrated consistent inhibition of aromatase activity by Roundup in various human cell lines. The concentrations required to cause these effects varied depending on the cell type and formulation but included from 4 mg/L in liver cells and 72 mg/L in placental and embryonic cells.[26−28] Less consistently, and to a smaller extent than Roundup, aromatase inhibition by glyphosate alone has also been reported (approximately 10-fold higher concentrations).[28] Additionally, both glyphosate and Roundup were reported to reduce testosterone production in rat testicular cells at concentrations from 0.36 mg/L,[29] but it is difficult to relate these results to the potential effects of these chemicals *in vivo*. Few studies have investigated the effects of glyphosate and its commercial formulations on the endocrine system *in vivo*. Drakes treated with 5 and 100 mg Roundup/kg (body weight) exhibited reduced levels of testosterone, corresponding with alterations in testis structure. In rats, maternal and juvenile treatment from 5 and 50 mg/kg (body weight) of Roundup impaired male reproductive development, with effects including alteration in testis structure, sperm production, and sex steroid production.[30−32]

Reproductive effects of Roundup and glyphosate in fish have seldom been investigated and are far from clear. While no evidence of altered gonadal development was evident in juvenile stickleback exposed to 0.1−100 μg/L glyphosate,[33] treatment with 3.6 mg/L Roundup had some negative impact on offspring production in Silver catfish.[34] The mechanisms contributing to this reproductive effect have not been investigated.

Given the extensive usage of glyphosate based herbicides, there is a clear potential for the environmental exposure of fish populations to glyphosate together with associated formulation products, which may modify its toxicity. This study aimed to examine the effects of Roundup formulation on reproduction in fish and to determine to what extend these effects were associated with the toxicity of glyphosate alone. To do this, we conducted a 21 day reproductive test in breeding colonies of zebrafish, to determine if reproduction, embryo development and embryo survival, were affected by exposure to 0.01, 0.5, and 10 mg/L (glyphosate acid equivalent) Roundup and 10 mg/L glyphosate. The two lower Roundup concentrations included in this study were chosen to represent concentrations that can be expected to occur in the environment regularly (0.01 mg/L) and during occasional peak contamination events (0.5 mg/L). The highest concentration tested (10 mg/L) is unlikely to occur in surface waters, and was included to facilitate the analysis of the mechanisms of toxicity. We included a treatment group exposed to 10 mg/L glyphosate alone to allow for a direct comparison of its mechanisms of toxicity with the equivalent a.e. concentration of Roundup. We hypothesized that the mechanisms of toxicity resulting in effects on reproduction might include oxidative stress and disruption of steroid biosynthesis, and to investigate this we conducted transcript profiling of a suite of genes involved in these processes in the gonads.

MATERIALS AND METHODS

Fish Maintenance. Colonies of 4 male and 4 female adult (20 week old) WIK strain zebrafish were established in individual 15 L glass tanks and allowed to breed naturally during a 7 day acclimation period. Fish were maintained according to Paull et al.,[35] and a full description of husbandry procedures is provided in the Supporting Information.

Chemical Exposures. Chemical exposure was conducted via a flow through system for a period of 21 days in accordance with OECD guidelines for fish reproductive tests, preceded by a 10 day pre-exposure period.[36] The treatment groups consisted of three concentrations of Roundup; 0.01, 0.5, and 10 mg/L glyphosate acid equivalent (using Roundup GC liquid glyphosate concentrate containing 120 g/L glyphosate acid, Monsanto, Cambridge, U.K.), 10 mg/L glyphosate (analytical grade; Molekula, Wimborne, U.K.), and a control group. Each treatment group was comprised of three replicate breeding colonies (4 males and 4 females) in 15 L tanks. Water samples were collected from each tank on days 7, 14, and 21 of the exposure period and stored at −20 °C prior to chemical analysis. Details of the analytical chemistry procedures are provided in Supporting Information.

Reproductive Test and Embryo Exposures. Group spawning occurred daily at dawn and eggs were collected 1 h post fertilization (hpf), rinsed thoroughly to remove detritus and incubated in water containing the same chemical exposure concentrations as their tank of origin, at 28 °C. Exposure water for the embryo experiments was made according to the ISO 7346-3:1996 guidelines,[37] fully oxygenated and supplemented with 2.5 μL/L of the antifungal agent methylene blue (Interpet, Dorking, U.K.) to avoid mortalities caused by fungal infections. The eggs from each colony were examined using light microscopy between 2 1/2 and 3 1/2 h after dawn, when all fertilized eggs had reached at least the 16-cell stage during early cleavage,[38] and the total number of fertilized and unfertilized eggs were quantified on each day throughout the pre-exposure and exposure periods. During the 21-day chemical exposure, fertilized eggs displaying cellular necrosis were counted and recorded as early stage mortalities (<3.5 hpf). Fifty fertilized eggs from each tank were selected randomly and incubated in 50 mL exposure water until 72 hpf. During this period, embryo mortality was recorded at 24, 54, and 72 hpf and embryo hatching was recorded at 54 and 72 hpf.

To determine if the observed effects of Roundup and glyphosate on embryos were because of the effects of exposure during gametogenesis or during embryogenesis, embryos collected from a control population were exposed to a range of concentrations of glyphosate and Roundup as above.

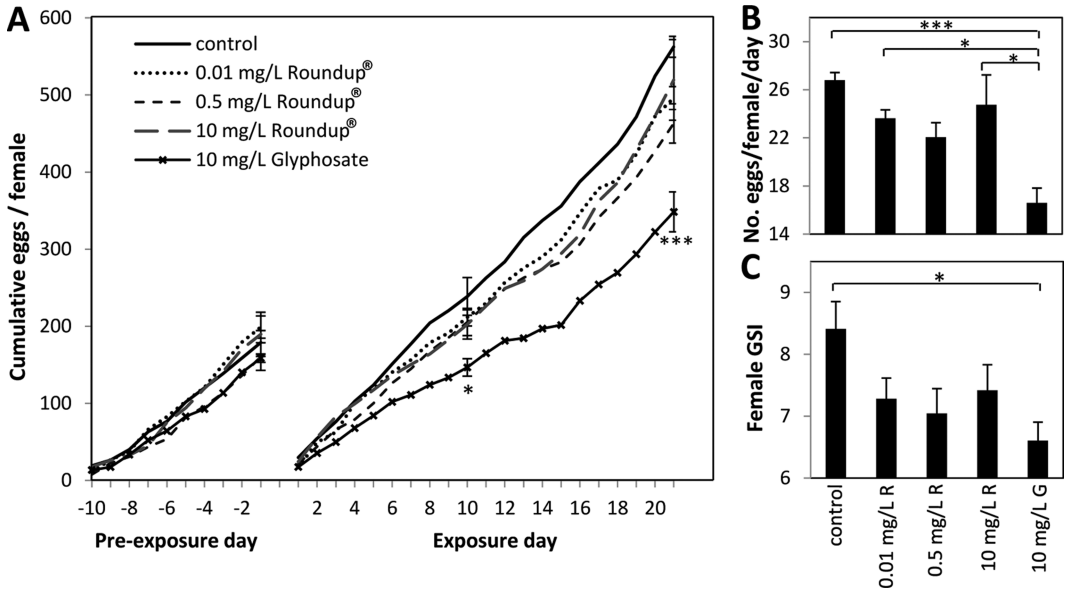

Figure 1. (A) Cumulative egg production during the 10 day pre-exposure and 21 day chemical exposure periods (n = 3 replicate colonies per treatment). (B) Mean number of eggs laid per female per day throughout the 21 day exposure period (n = 3 replicate colonies per treatment), and (C) mean gonad-somatic index of females in each treatment group (n = 12 individual females per treatment). Data plotted are mean values ± SEM. Asterisks indicate significant differences between treatment groups (*P < 0.05, **P < 0.01, ***P < 0.001).

Chemical treatment was initiated between 10 and 20 min post fertilization. In addition to the exposure concentrations used for the adult exposures, embryos were also treated with higher concentrations (50, 100, 250, 500, and 1000 mg/L a.e. Roundup and glyphosate) to determine the concentration thresholds for embryo mortalities and developmental toxicity. Experiments were conducted in triplicate; each replicate contained 50 embryos and observations of mortalities and hatching were performed as described above.

Sampling. All fish were humanely sacrificed on day 21 of the exposure period by a lethal dose of benzocaine (0.5 g L^{-1}; Sigma-Aldrich) followed by destruction of the brain, in accordance with U.K. Home Office regulations. Wet weight and fork length were recorded and the condition factor (k = (weight (g) × 100)/(fork length $(cm)^3$)) was calculated for individual fish. Livers were dissected and weighed, and the hepatosomatic index (HSI) (liver weight (mg)/total weight (mg)) × 100)) was determined for individual fish. Gonads were dissected, weighed and one gonad from each fish was snap frozen in liquid nitrogen and stored at −80 °C prior to transcript profiling. The remaining gonad was fixed in Bouin's solution (Sigma-Aldrich) for histological analysis. The gonadosomatic index (GSI; gonad weight (mg)/total weight (mg)) × 100)) was determined for both males and females.

Transcript Profiling and Histological Analysis. Transcript profiling of genes encoding steroidogenic enzymes, sex steroid receptors and antioxidant enzymes, was conducted using RT-QPCR in the gonads of exposed fish according to ref 39. Histological analysis of the gonads was conducted according to ref 40. A full description of these methodologies is presented in the Supporting Information.

Statistical Analysis. Statistical analyses were conducted with SigmaStat (version 12.0). Before analysis, proportional data (embryo survival and hatching) were subjected to variance-stabilizing square-root or arcsine transformations as appropriate. All reproductive output and sampling data met assumptions of normality and equal variance. Outliers in transcript expression data were identified and removed according to Chauvenet's criterion[41] prior to statistical analysis. Transcript expression data that did not meet normally distributed criteria was log transformed before statistical analysis. All data was analyzed using single factor one way analysis of variance (ANOVA), followed by the Holm−Sidak post hoc test using a pairwise comparison method. Data were considered to be significant when P < 0.05.

RESULTS

Water Chemistry. The mean measured concentrations of glyphosate in the tank water were between 88% and 140% of the nominal values for all treatments (quantification of glyphosate in tanks receiving 0.01 mg/L Roundup was below the detection limit of our method) and are presented in Supporting Information Table S2.

Morphometric Parameters. The mean mass and length of male and female fish were 375.0 ± 6.3 mg/32.6 ± 0.2 mm and 402.6 ± 9.3 mg/31.7 ± 0.2 mm, respectively. There were no significant differences in size or condition factor (mean 1.08 and 1.25 for males and females, respectively) between treatment groups. Additionally, we observed no alteration of general health or behavior in any colony. The GSI of females was significantly lower in the fish treated with 10 mg/L glyphosate compared to the control group (Figure 1c). There was no significant difference in the GSI of males between treatment groups, or in the HSI of males or females.

Reproductive Test and Embryo Exposures. During the 10 day pre-exposure period, there was no difference in cumulative egg production between the treatment groups (P = 0.468). During the exposure period, colonies in the control group consistently spawned the greatest number of eggs per female, while those treated with 10 mg/L glyphosate spawned the least. From day 10 of the exposure period, cumulative egg production was significantly reduced in colonies exposed to 10 mg/L glyphosate compared to the controls, and this difference intensified throughout the remainder of the exposure period. At

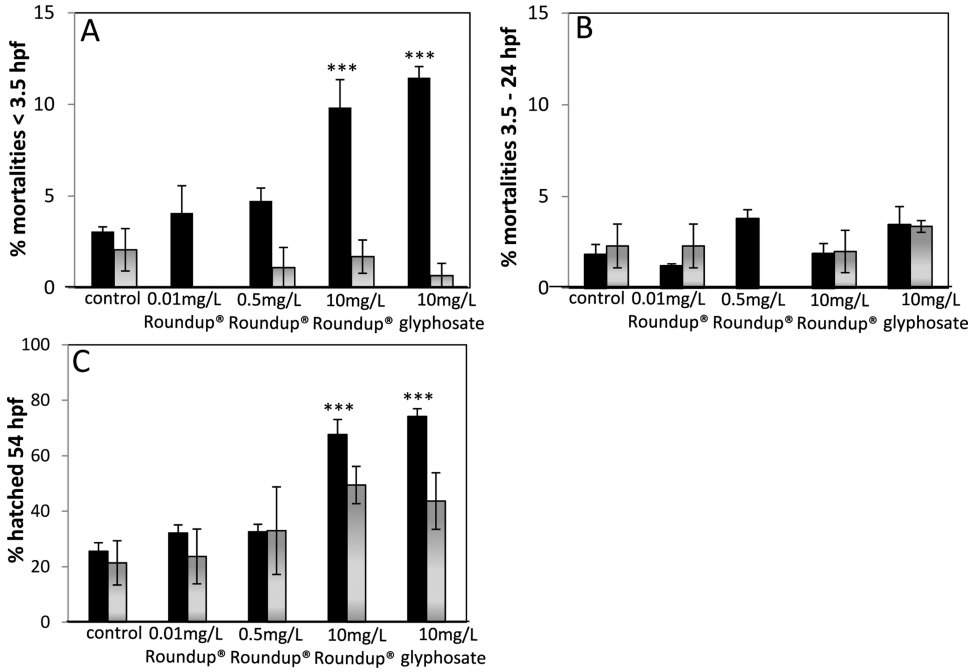

Figure 2. Effects of Roundup and glyphosate on embryo survival and development. Black bars represent embryos originating from exposed parental populations (n = 3 replicate colonies, for each colony data was collected every day for 21 days of exposure and averaged) and gray bars represent embryos originating from a control parental population (n = 3 replicate exposures, each replicate containing 50 embryos). (A) Percentage of embryo mortalities that occurred before 3.5 hpf. (B) Percentage of embryo mortalities that occurred between 3.5 and 24 hpf. (C) Percentage of embryos that had hatched at 54 hpf in each treatment group. Data plotted are mean values ± SEM. Asterisks represent significant differences from the control treatment (***$P < 0.001$).

the end of the 21 day exposure, cumulative egg production was significantly lower in colonies exposed to 10 mg/L glyphosate compared to the control, and also compared to the 10 and 0.01 mg/L Roundup groups (Figure 1a,b). Additionally, egg output significantly correlated (R^2 = 0.79; P = 0.043) with female GSI across all treatment groups. Fertilisation rate remained consistently high throughout the exposure period with no significant differences between treatment groups and an overall mean value of 83.4%.

There was a significant increase in embryo mortalities occurring before 3.5 hpf in embryos from both the 10 mg/L Roundup and glyphosate treatment groups (Figure 2a). Additionally, there was a significant correlation between early embryo mortality and the concentration of Roundup (R^2 = 0.52; P = 0.008). There were no significant differences between treatments in embryo mortality between the start of epiboly (3.5 hpf) and the end of somitogenesis at 24 hpf (Figure 2b). However, there was a significant increase in the percentage of embryos that had hatched at 54 hpf in groups treated with 10 mg/L Roundup and 10 mg/L glyphosate compared to the control group (Figure 2b).

For embryos originating from a control population, exposure to glyphosate and Roundup at the concentrations used in the adult reproductive test (0, 0.01, 0.5, and 10 mg/L Roundup and 10 mg/L glyphosate) did not result in increased mortality rate at either 3.5 hpf or 24 hpf (Figure 2a,b), but there was a significant increase in 3.5−24 hpf mortality in embryos exposed to concentrations ≥100 mg/L glyphosate and ≥500 mg/L Roundup (Supporting Information Figure S4a). We also observed evidence of developmental delay and abnormalities from concentrations ≥50 mg/L glyphosate and ≥250 mg/L Roundup at 24 hpf. There was a trend toward increased hatching at 54 hpf in groups exposed to 10 and 50 mg/L Roundup and glyphosate, and there was a significant correlation between hatching rate at 54 hpf and exposure concentration of Roundup up to 50 mg/L (R^2 = 0.27; P = 0.04) (Supporting Information Figure S4b). For embryos exposed to ≥100 mg/L Roundup and glyphosate, we found evidence of progressive delay in development and hatching with increasing concentration.

Gonad Transcript Profiling. In the ovary, the transcript encoding aromatase (*cyp19a1*) was significantly up-regulated in the 10 mg/L Roundup treatment group compared to the controls. Estrogen receptor 1 (*esr1*) in the 10 mg/L Roundup group was significantly up-regulated compared to the 10 mg/L glyphosate group. There were similar, but not statistically significant, decreasing trends in expression of other steroidogenic enzymes including cytochrome P450, subfamilies 17 and 11 (*cyp17a1*, *cyp11a1*) and 3β-hydroxysteroid dehydrogenase (*hsd3b2*) in groups exposed to both Roundup and glyphosate. In contrast, for the antioxidants glutathione peroxidase (*gpx1a*), catalase (*cat*) and glutathione-S-transferase pi (*gstp1*) non-significant, increasing trends in transcript expression were observed (Figure 3a, Supporting Information Figure S1a).

In the testis, *hsd3b2* was significantly up-regulated following exposure to 10 mg Roundup/L compared to all other treatment groups. The expression pattern of steroidogenic acute regulatory protein (*star*), *cyp17a1*, *cyp11a1*, and the androgen receptor (*ar*) additionally appeared to follow an expression pattern similar to *hsd3b2* across treatment groups. *cat* was significantly up-regulated in groups exposed to both 10 mg/L Roundup and 10 mg/L glyphosate compared to those treated with 0.5 mg/L Roundup. In addition, *sod1* was significantly up-regulated in the 10 mg/L compared to 0.5 mg/L Roundup groups (Figure 3b, Supporting Information Figure S1b).

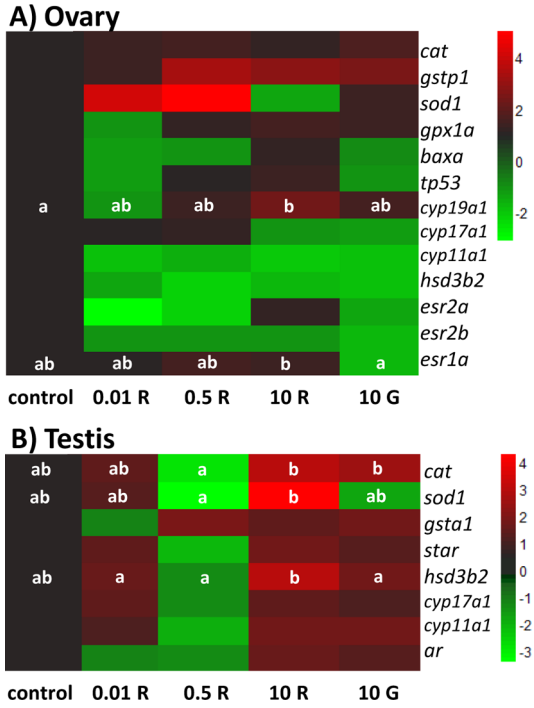

Figure 3. Transcript profiling of target genes in the ovary (A) and testis (B) following exposure to Roundup (R) and glyphosate (G). Data are presented as fold change relative to expression in the control group, whereby red shading indicates up-regulation and green shading represents down-regulation. Relative expression was calculated as ratio of target gene/rpl8 mRNA concentration. For each treatment, $n = 6–8$ fish. Individual data points classified as outliers, and for which the expression was below the detection limit of the assay were excluded from the analysis. Lettering indicates significant differences between treatment group, with groups identified with different letters being significantly different from each other ($P < 0.05$).

Gonad Histology. Histological examination of females from all treatment groups showed that the ovaries of all individuals contained oocytes at all stages of development (oogonia, primary oocytes, cortical alveoli stage oocytes, secondary oocytes, and mature vitellogenic oocytes) and the majority contained recent postovulatory follicles. We found evidence of ovarian abnormalities in 9.1%, 18.2%, 9.1%, 50.0%, and 63.6% of females in the control, 0.01 mg/L Roundup, 0.5 mg/L Roundup, 10 mg/L Roundup and 10 mg/L glyphosate treatment groups, respectively (Supporting Information Figure S3). The majority of abnormalities were relatively mild and included accumulation of eosinophilic fluid and presence of abnormal tissue. In addition, the proportion of fish containing atretic oocytes in their ovaries also appeared to be increased (Supporting Information Figure S2).

Histological examination of males showed that testes of all individuals from all treatment groups contained germ cells at all stages of spermatogenesis (including spermatogonia, spermatocytes, spermatids, and mature spermatozoa) (Supporting Information Figure S2). There were no abnormalities and no differences between stages of development between treatment groups.

DISCUSSION

Reproductive Effects on Adult Zebrafish. This study provides evidence that glyphosate caused a reduction in the number of eggs spawned by female zebrafish exposed to high concentrations (10 mg/L) of glyphosate. However, this concentration is well above concentrations measured to date in the environment and unlikely to occur in aquatic systems, except when glyphosate is directly applied to control algal populations. In addition, our study also showed an apparent reduction, albeit not significant, in egg production in all three Roundup treated groups. Therefore, the potential for adverse effects of Roundup on reproductive output and impact on wild populations cannot be ruled out. A number of potential mechanisms may contribute to the observed effect of glyphosate on egg production, including disruption of normal progression through oogenesis, inhibition of ovulation and increased rate of oocyte atresia. To explore this, we conducted histological analysis of the gonads of exposed females and observed a trend toward an increase in the incidence of ovarian abnormalities as a result of exposure to both Roundup and glyphosate. Ovarian follicle atresia is an apoptotic process leading to reabsorption of maturing oocytes rather than ovulation. It is a highly regulated, natural process thought to have a role in maintaining ovarian homeostasis; however various environmental stressors, as well as disruption of the hormonal control of oogenesis and ovulation, have been shown to increase atresia.[42] We found atretic vitellogenic oocytes in all treatment groups, but this incidence tended to increase in both the 10 mg/L Roundup and 10 mg/L glyphosate treatment groups. Similarly, in these groups we also found an increased trend in the incidence of abnormal ovarian tissue, including excess connective tissue and putative hemopoietic tissue. In some females treated with 0.01 and 10 mg/L Roundup and 10 mg/L glyphosate, we observed the presence of areas containing eosinophilic fluid. Previously, accumulated proteinaceous fluid in the ovary has been found to contain vitellogenin, and this has been associated with a disruption in the endocrine control of oogenesis in zebrafish through exposure to elevated levels of 17β-oestradiol.[43]

It is important to note that despite the trends toward increased incidence of atretic follicles and ovarian abnormalities following exposure to glyphosate and Roundup, the majority of fish were only moderately affected and their ovaries contained oocytes at all stages of maturation, including mature vitellogenic oocytes and postovulatory follicles. Moreover, we found no differences in the ovarian expression of bcl2-associated X protein (*baxa*) and tumor protein 53 (*tp53*), which are typical marker genes of apoptosis. This indicates that oocyte atresia was unlikely to be the major mechanism responsible for the decline in egg production rate induced by glyphosate treatment. Corresponding with this, a similar degree of atresia in fish exposed to Roundup was not accompanied by a significant decline in egg-production in this treatment group. Therefore, we hypothesize that the observed decrease in egg production following exposure to glyphosate was more likely to be due to a reduction in the number of follicles undergoing oogenesis. The strong correlation between egg production and female GSI, including a significant reduction in GSI in females exposed to 10 mg/L glyphosate, which indicates reduced gonadal volume, provides support for this hypothesis.

Sex steroids are essential for the regulation of oogenesis, and alterations in sex steroid biosynthesis may have contributed to the reduction of egg production in colonies exposed to glyphosate. To test this hypothesis, we investigated the effects of glyphosate and Roundup on the expression of a number of transcripts encoding enzymes involved in steroid biosynthesis, several of which have previously been shown to be targets of

their toxicity.[25−28] We found a significant increase in the expression of ovarian aromatase, an enzyme which catalyzes the conversion of testosterone to estradiol in granulosa cells, in the gonads of females exposed to 10 mg/L Roundup, and also an increasing trend in those exposed to 10 mg/L glyphosate. Several previous studies have demonstrated that Roundup disrupts both aromatase activity and *cyp19a1* expression levels in a number of human cell lines, and there is some evidence that glyphosate can also inhibit aromatase activity, especially with the addition of small percentages of Roundup, which may facilitate its cellular entry.[26−28] Romano et al.[32] proposed inhibition of aromatase as a causative mechanism for disruption of steroidogenesis and adverse reproductive impacts in the male offspring of rats exposed to Roundup during pregnancy. The stimulatory effect of Roundup on *cyp19a1* expression observed in the present study contrasts with the predominantly inhibitory effects found in the *in vitro* studies. This may reflect the complex nature of feedback mechanisms governing steroid biosynthesis pathways *in vivo* or, possibly, a compensatory transcriptional response to a potential inhibition of aromatase enzyme. Additionally, it is difficult to equate the concentrations used in the present study with those used in the *in vitro* studies. It is possible that differential stimulatory and inhibitory responses occur with concentration, and also with time. Although not significant, there were also similar decreasing trends in expression of steroidogenic enzymes, *hsd3b2, cyp17a1* and *cyp11a1,* in females treated with both 10 mg/L of Roundup and 10 mg/L glyphosate, indicating a possible wider effect on steroidogenic pathways.

The differential regulation of ovarian *esr1* by Roundup and glyphosate is interesting and may reflect the effect of other chemicals present in Roundup formulation on this receptor. Increased *esr1* expression following Roundup exposure may have resulted from compensatory mechanisms in the ovary to maintain or restore estrogen signaling pathways. This may explain, at least in part, the differences in the effects of these chemicals on egg production, with glyphosate having a more pronounced effect than Roundup. Using human liver HepG2 cells, Gasnier [26] showed that Roundup and glyphosate antagonistically bind estrogen receptors (*ERα* and *ERβ*), although Kojima [44] found no evidence of agonistic or antagonistic interaction with estrogen receptors in Chinese hamster ovary cells. A recent study showed glyphosate actively bound estrogen receptors and induced proliferative growth of estrogen-dependent breast cancer cells, and also increased protein levels of ERα and ERβ.[45] Taken together, our ovarian transcript profiling data suggests that Roundup and glyphosate may have disrupted steroid hormone biosynthesis and also potentially modulated the biological effects of estrogens via alterations in the expression of *esr1*, the predominant estrogen receptor in the ovary.

Despite having no significant effect on egg production, it is interesting to note that exposure to 10 mg/L Roundup also elicited alterations in gene expression often in the opposite direction of those induced by exposure to the equivalent concentration of glyphosate alone. This might suggest the presence of compensatory mechanisms ameliorating the adverse effects of glyphosate when in the presence of the other constituents of Roundup. A possible mechanism could be increased synthesis of aromatase to maintain sex steroid ratios and estrogen signaling in the ovary in order to promote oogenesis, and maintain egg production.

There was no effect of exposure to Roundup or glyphosate on fertilization rate. Corresponding with this, histological examination revealed no evidence of any disruption of spermatogenesis, or abnormalities in the testis following exposure to glyphosate or Roundup. Therefore, we found no indication that these chemicals affect the ability of the sperm produced to fertilize eggs. This contrasts with several previous *in vivo* studies that have found some evidence that Roundup disrupts spermatogenesis in rats, resulting in testis pathology, sperm abnormalities and altered sperm production.[30−32] It is important to note, however, that our experimental conditions are optimized to maximize reproduction and may not detect subtle changes in sperm quality that may be sufficient to cause effects under the conditions found in the natural environment.

Previous Roundup-induced testicular toxicity has been associated with alterations in steroidogenesis and sex steroid levels in rats and drakes.[30−32,46] In the current study, analysis of transcripts encoding steroidogenic enzymes in the testes showed that *hsd3b2* was significantly up-regulated in males exposed to 10 mg/L Roundup compared to those exposed to 0.01 and 0.5 mg/L Roundup, and 10 mg/L glyphosate (Figure 3b). Moreover, although not statistically significant, the expression patterns of the other steroidogenic enzymes profiled (*star, cyp17a1,* and *cyp11a1*), as well as *ar*, followed a similar expression pattern to *hsd3b2* across treatment groups. This pattern, of apparent down-regulation in the 0.5 mg/L Roundup treatment and up-regulation in the 10 mg/L Roundup group was robust across tank replicates. Walsh et al.[25] found evidence that Roundup, but not glyphosate, disrupted StAR and P450scc (Cyp11a1) in mouse testis cells, primarily through alteration of protein expression and activity, suggesting that such post-transcriptional regulatory changes should also not be ruled out. Additionally, we found 10 mg/L Roundup significantly increased expression of *cat* and *sod1* compared to the lower Roundup treatments, and 10 mg/L glyphosate also significantly increased *cat* expression in the testis. Together, these changes in the transcription of antioxidant enzymes provide evidence that both Roundup and glyphosate induce oxidative stress in the testis.Therefore, despite no apparent impacts on fertilization success, we have found some evidence that high concentrations of Roundup and glyphosate cause disruption of steroidogenesis and oxidative stress in the testis, suggesting that their potential to cause adverse impacts on male reproductive health should not be ruled out. It is interesting to note that exposure to 10 mg/L Roundup elicited differential responses, in terms of the magnitude and direction of transcript expression changes, compared to 10 mg/L glyphosate, possibly suggesting greater compensatory mechanisms of response following exposure to Roundup, similarly to that observed in females.

Effects on Embryo Survival and Development. We found evidence that treatment with both 10 mg/L Roundup and glyphosate induce an increased rate of embryo mortality during very early development. We observed necrosis of the fertilized embryos during cleavage and early blastula stages, prior to progression to epibioly at ∼3.5 hpf (as described by Kimmel et al.[38]). To assess if the early stage mortality was caused as a direct result of the chemical exposure on embryos or by the parental exposure, we exposed embryos originating from a control population of untreated adults and found that concentrations of up to 10 mg/L of Roundup and 10 mg/L glyphosate had no effect on embryo survival at <3.5 or 3.5−24 hpf. This corresponds with previous work showing exposure of

zebrafish embryos to up to 10 mg/L glyphosate for 5 days had no effect on survival or development.[47] We only found a significant increase in embryo mortality at concentrations of 100 mg/L glyphosate and 1000 mg/L Roundup, which are 10 and 1000 times higher than the concentrations used in the reproductive study. Moreover, this mortality predominantly occurred between 3.5 and 24 hpf, rather than in the earlier stages of development. These high concentrations of glyphosate, and to a lesser extent Roundup formulation, result in a pronounced decrease in pH in the exposure water (to 3.8 (100 mg/L glyphosate) and 4.9 (1000 mg/L Roundup)), which may be responsible for the embryo toxicity seen. Overall, these results suggest that the increase in early stage mortalities observed in embryos originating from fish exposed to 10 mg/L Roundup and glyphosate is attributable to potential damage of the gametes occurring during gametogenesis and/or fertilization, rather than as a result of direct embryo exposure. Alternatively, it is possible that maternal transfer of glyphosate, Roundup or formulation products, via the yolk, might contribute to embryo exposure to these toxicants and the increased mortality observed.

As discussed above, gonadal transcript profiling revealed significant up-regulation of transcripts encoding antioxidant enzymes in response to exposure to 10 mg/L Roundup and 10 mg/L glyphosate in the testes and increasing trends in transcripts encoding antioxidant enzymes in the ovary. Oxidative stress induced in the testis by chemical exposure has been shown to cause DNA damage in developing sperm.[48] Pérez-Cerezales et al.[49] showed that DNA damage in rainbow trout sperm did not impair fertilization success, but resulted in a high rate of embryo mortality in early stages of embryogenesis, particularly during gastrulation. This is consistent with our findings that fertilization success was unaffected but that an increased rate of embryo mortalities occurred during early stages of development and before transition to epiboly. Therefore, we hypothesize that oxidative stress generation in the testis during spermatogenesis is likely to be an important causative mechanism responsible for the increase in early stage embryo mortality. Additionally, the increase in ovarian histological abnormalities and the increased trends in ovarian antioxidant transcript expression suggest similar damage during oogenesis is also possible, although oocytes are thought to have greater response and repair mechanisms to counter-act oxidative stress than sperm.[49,50] DNA damage after spermiation cannot be ruled out, but probably has a minimal effect compared to damage during spermatogenesis, given the brief period of less than 65 s that sperm remains motile before fertilization (Van Look et al., personal communication).

We found an increased percentage of hatching at 54 hpf in groups exposed to 10 mg/L Roundup and 10 mg/L glyphosate. Additionally, embryos originating from the unexposed control population showed a significant increasing trend in hatching rate at 54 hpf with concentrations up to 50 mg/L Roundup, as well as an apparent increase in hatching in those treated with 10 mg/L glyphosate. This suggests an independent impact of Roundup and glyphosate on embryos, not entirely attributable to toxicity during gametogenesis. Hatching is variable, and dependent on a number of environmental factors. Various chemical and other environmental stressors, such as temperature, are known to affect developmental rate and, subsequently, time to hatch. However, in this study, observations at 24, 48, 54, and 72 hpf showed no obvious change in development rate between treatment groups, indicating that exposure to 10 mg/L Roundup and 10 mg/L glyphosate induces premature hatching in zebrafish. At 72 hpf, more than 90% of embryos from all treatment groups had hatched (both those originating from exposed and nonexposed adults), and there were no obvious behavioral or morphological differences between treatments. In natural populations, premature hatching could potentially result in detrimental impacts for population sustainability, for example by increasing the susceptibility to predation.

We found no obvious signs of developmental toxicity at exposure concentrations up to 10 mg/L Roundup or glyphosate, which corresponds with the findings of Stehr.[47] We did find evidence of developmental delay in embryos exposed to concentrations ≥50 mg/L glyphosate and ≥250 mg/L Roundup and hypothesize that the increased toxicity of glyphosate may be attributed to its greater acidity than the buffered Roundup formulation. With the exception of amphibians, which appear particularly sensitive [e.g., refs 23 and 24], these results show that only extremely high concentrations of Roundup and glyphosate induce developmental toxicity in zebrafish and are generally in accordance with evidence from other species, including rats[51] and sea urchins.[52]

Overall, we have found evidence that both 10 mg/L Roundup and 10 mg/L glyphosate have similar adverse impacts on embryo survival and hatching, while 10 mg/L glyphosate reduces egg production. We have found some evidence that these reproductive effects occur via multiple mechanisms of toxicity which appear to differ, to some extent, between Roundup and its active ingredient glyphosate. These mechanisms may include disruption of the steroidogenic pathway and sex steroid signaling, and generation of oxidative stress. This work demonstrates that both glyphosate and Roundup have a detrimental impact on a number of measures of reproductive health in zebrafish, although only at very high concentrations that are unlikely to occur in the environment, based on the currently available measurements. Given the growing concern over potential reproductive effects of these compounds, and their extremely widespread usage, this provides valuable mechanistic information for their environmental risk assessment, particularly when considering the potential effects of complex mixtures of environmental contaminants.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental experimental section, target genes, primer sequences, and assay details for RT-QPCR analysis, measured concentrations of glyphosate in tank water, transcript profiling of target genes in the gonads, gonad histology of control and exposed fish, occurrence of ovarian histological abnormalities, effects of glyphosate and Roundup on embryos originating from a control population. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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