

Disturbances in reproduction and expression of steroidogenic enzymes in aquatic invertebrates exposed to components of the herbicide Roundup

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Abstract

Exposure of organisms to environmental contaminants is a growing concern. We have investigated the effects of the individual active ingredients of the herbicide Roundup (glyphosate and diquat dibromide [DD]) since Roundup causes alterations in reproduction, mortality, and development in the aquatic snail *Lymnaea palustris*. Snails chronically treated with elevated but ecologically relevant levels of DD exhibit reduction in fecundity ($p < 0.05$), while fecundity in glyphosate-treated snails is comparable to or exceeds control levels. To investigate a possible mechanism for the reproductive disturbance, we monitored levels of steroid acute regulatory (StAR) protein in whole snails and observed a correlation in StAR protein decrease with treatment with Roundup, glyphosate, or DD. We detect StAR in organs where steroid biosynthesis occurs (ovotestis, brain, kidney); StAR protein is reduced following chronic exposure to Roundup, glyphosate, or DD ($p < 0.01$). Estradiol and testosterone concentrations in hemolymph were measured by enzyme-linked immunosorbent assay following 3-week exposure of snails to 3.5 mg/L glyphosate or 140 μ g/L DD. Testosterone levels decrease in DD-treated groups ($p < 0.05$); a trend of lower testosterone is also observed in glyphosate-treated groups ($p > 0.05$). Estradiol concentration is greater than or equal to control levels in glyphosate, but decreased in DD ($p < 0.05$). Because of its role in the conversion of testosterone to estradiol, we monitored abundance of aromatase and observed a reduction ($p < 0.05$) in DD-treated snails (consistent with the drop in fecundity and estradiol levels) and a comparable level to control in glyphosate-treated snails (consistent with their high fecundity and estradiol levels). Although the toxicity of commercially-available Roundup to aquatic animals may have many contributing factors including its inactive surfactant, the constituent of Roundup associated with the greatest reproductive disturbances and observed developmental abnormalities of offspring is DD. This study details the analysis of particular herbicide constituents and their effect on specific targets in the reproductive pathway.

Keywords

Toxicology, reproduction, *Lymnaea*, Roundup, snails, steroidogenesis

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Introduction

An abundance of herbicides and pesticides in the environment poses an increasing threat to nontarget species. These chemicals may reach freshwater habitats by surface runoff, groundwater contamination, or aerial drift. Roundup is a widely-used and highly water-soluble herbicide. Its main active ingredient, glyphosate, has been widely studied *in vitro*,^{1,2} though far less extensively in whole organisms.

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In some studies, glyphosate was found to have relatively low toxicity in isolation,³ although developmental and reproductive disturbances have been documented in invertebrate⁴ and vertebrate⁵ aquatic and terrestrial⁶ organisms; additionally, its bioaccumulation and health effects have recently raised alarm.⁷ Studies testing the effects of Roundup's surfactant additive polyethoxylated tallowamine (POEA⁸) have demonstrated that other components contribute to greater toxicity than glyphosate alone,^{2,9–11} and researchers¹² have suggested that “inert” adjuvants’ contribution to toxicity has been drastically underestimated. Preliminary findings on exposure of our invertebrate model *Lymnaea palustris* to Roundup or its individual constituents suggested that—while POEA (32.5 µg/L) exerted the highest mortality, and glyphosate plus POEA resulted in increased size of egg clutches¹³—another component was responsible for the highest suppression of fecundity and rise in developmental abnormalities. Our research has determined that diquat dibromide (DD) is capable of causing those effects.

DD is a broad-spectrum herbicide, often used in combination with other herbicides. Due to the rapid dissipation of diquat in water¹⁴ and subsequent binding to sediment and soil, DD has been assumed safe for direct application to water systems to control aquatic weeds, and US EPA Tolerance Reassessment Progress and Risk Management Decision (TRED) reports indicate no harm will result from exposure to DD within established and proposed tolerances, up to 2 mg/L for fish and 20 mg/L for shellfish. This has led to increased exposure of freshwater animals to DD, organisms for which the effects of this compound are largely uncharacterized.¹⁵ Depending upon application time, proximity to agricultural areas, and rainfall, runoff to surface water causes Roundup concentration to regularly equal or exceed the US EPA recommended maximum contaminant level (MCL) of glyphosate for human drinking water (0.7 mg/L) as well as the maximum contaminant level goal (MCLG) of DD (0.02 mg/L) in many regions of the United States and worldwide.^{7,16,17} Studies have documented altered fecundity, developmental delays,¹⁸ and altered transcriptional and enzymatic activity of markers of oxidative stress following acute DD treatment in *Lymnaea stagnalis*.¹⁹ While initial characterization of the molecular basis of an organism's response to estrogen²⁰ and biocides²¹ on the steroidogenic pathway and of herbicides on oxidative stress was made,¹⁹ none have characterized the basis of altered fecundity of molluscs in response to herbicides. We observed snails chronically treated in Roundup exhibited reduced fecundity and high embryonic abnormalities,¹³ found DD to be a potent developmental teratogen,²² leading to the investigation of the effects of Roundup's active ingredients on reproduction.

Since steroid acute regulatory (StAR) protein is the rate-limiting factor in steroidogenesis^{23,24} and has been shown to be disrupted in mammalian cells exposed to a variety of compounds, we sought to determine whether StAR

abundance in molluscs is altered *in vivo* under experimental conditions. Aromatase, a cytochrome P450 enzyme in the steroidogenic pathway, converts androgens to estrogens. This pivotal role makes it an enzyme of interest in determining toxicological effects upon hormones, gametogenesis, and reproductive health. We quantified testosterone and estradiol in exposed snails (Figure 1). Since these hormones play a major role in regulation of gametogenesis, alterations in their abundance may be linked to the observed changes in fecundity; chemicals that act as endocrine disruptors in vertebrates^{25,26} have been demonstrated to alter endogenous steroid hormone levels in hermaphroditic gastropods.²⁶ Additionally, fluctuations in sex steroid levels have been linked to the reproductive cycle in molluscs.²⁷

Lymnaea (Stagnicola) palustris is a Basommatophoran gastropod mollusc found worldwide in freshwater lakes, streams, and rivers. *Lymnaea* is the preferred genus to *Stagnicola*²⁹ for the Eurasian species *palustris*, which is very highly related to the North American *elodes*, and these have been considered conspecific.³⁰ *L. palustris* are hermaphroditic and suitable for use as ecotoxicological indicators due to hardiness, year-round reproductive output, rapid developmental progression, ease of rearing and laboratory testing,³¹ and high sensitivity to mutagens as documented in closely-related species.^{32,33} We applied DD and glyphosate and observed their effects on *L. palustris*. StAR, reproductive output, and steroid hormone levels are altered following exposure. Although Roundup exerts overall effects on development and survival in these aquatic snails, we find that specific active ingredients may be correlated with alterations in fecundity that may target components of the steroidogenic pathway.

Methods

Animal culture and treatment

Laboratory-reared *L. palustris* were housed in filtered aerated aquaria in artificial pond water (APW) and fed rinsed organic romaine lettuce *ad libitum*. Adult snails used in the study ranged from 1.4 cm to 1.8 cm in shell height; 400 mL covered mesocosms with aeration were used for all adult chronic treatments; snails were housed at four adults per unit on a 12:12 light–dark cycle at $21 \pm 1^\circ\text{C}$. Mesocosms were maintained with 100% change of solution twice weekly. Jelly masses containing fertilized eggs are oviposited by adults on tank walls or bottom (see Figure 2(b)). Jelly masses were harvested and total number of embryos per tank recorded twice weekly.

Three mesocosms were established for each of three treatment types ($n = 36$) and housed for 3 weeks: control APW, DD (140 µg/L), and glyphosate (3.5 mg/L), and repeated for a total $n = 72$. Other animals in parallel and pilot studies ($n = 108$) were established similarly during which snails were housed for up to 6 weeks; although guidelines are in place for 4-week³² chronic exposure times

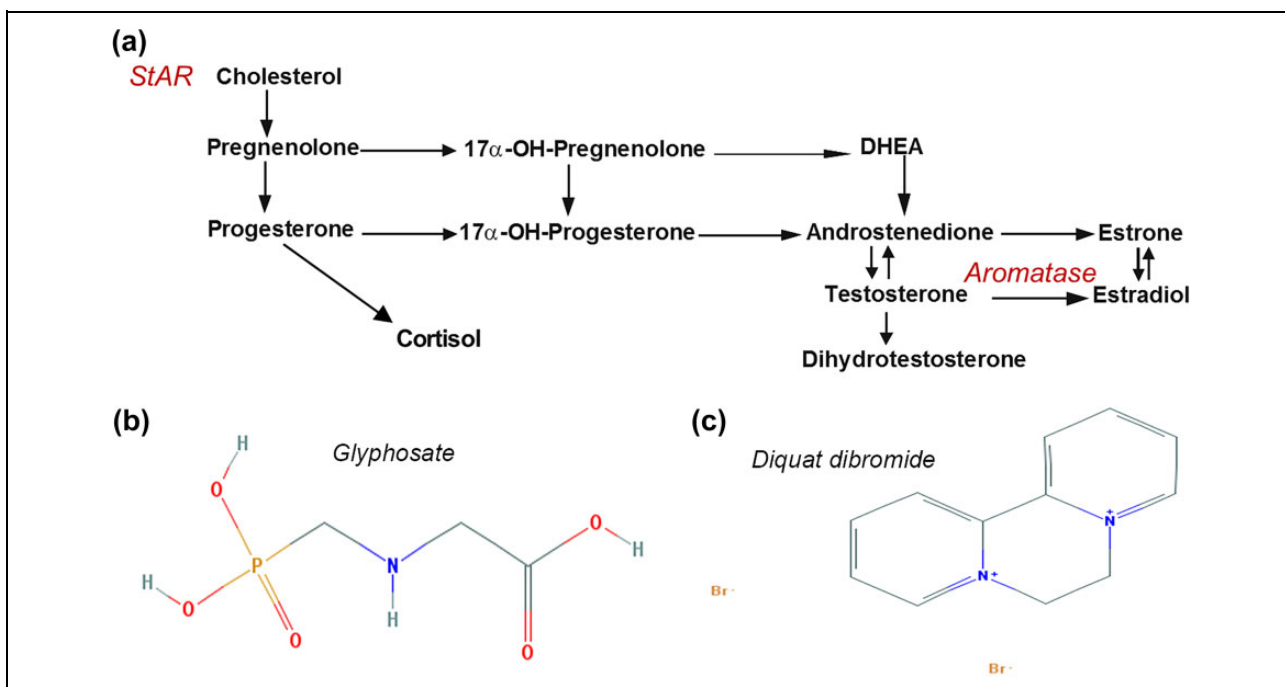


Figure 1. (a) Steroidogenic pathway in molluscs. StAR is the rate-limiting factor in steroidogenesis and ushers cholesterol to the inner mitochondrial membrane. P450-aromatase (CYP19 or aromatase) converts testosterone to estradiol. Panel adapted from Janer and Porte.²⁸ (b and c) Chemical structures of herbicides used in this study, generated by National Center for Biotechnology Information PubChem Compound Database: (b) structure of glyphosate and (c) structure of diquat dibromide. StAR: steroid acute regulatory protein.

of related species, 3 weeks of exposure is also common in pond snails²⁶ and consistently yields reproductive disturbance in our species.¹³ Animals were exposed to complete Roundup at 19.5 mg/L; the concentrations of DD and glyphosate solutions were set at five times the US EPA MCL (glyphosate) or MCLG (DD), or allowable concentration in drinking water (EPA 2018), and the Roundup concentration was based on five times the MCL of glyphosate. These concentrations, while in excess of human drinking water recommendations, are typically exceeded in surface waters for a period of days or longer following application of DD-¹⁷ or glyphosate-containing³⁴ herbicides.

Steroid analysis

Hemolymph was drawn weekly (once at outset of study, and following each of 3 weeks of treatment) from snails using a noninvasive, nonlethal method whereby the animal is induced to extrude hemolymph by a poke on the foot with a pipette tip.³⁵ Hemolymph was flash frozen in liquid nitrogen and stored at -80°C until analysis by enzyme-linked immunosorbent assay (ELISA). Hemolymph, approximately 50 μL per animal per draw, was pooled from all individuals in one mesocosm at a given time point. ELISA analyses for estradiol and testosterone were conducted and data analyzed according to manufacturer's instructions (Cayman Chemicals, Ann Arbor, MI 582701, 582251). Absorbance of samples and standards was read in BioTek, Winooski, VT

Synergy HT Multi-Detection Microplate Reader and calculations made in Microsoft Excel ($r^2 > 0.995$).

Protein analysis

To analyze differential aromatase abundance, ovotestes and associated structures (prostate and albumen glands) were harvested at the end of the study period. Tissues were mechanically disrupted in RIPA buffer (ThermoFisher, Waltham, MA 89900) containing protease inhibitor cocktail (ThermoFisher 78425) according to manufacturer's directions, and snap frozen in liquid nitrogen followed by storage at -80°C prior to analysis by Western blot. Samples were *O*- and *N*-glycosidased (New England Biolabs, Ipswich, MA E0540 S), approximately 1–10 μg per sample (at fixed volume) run under denaturing conditions on 12% polyacrylamide gel electrophoresis, and transferred using iBlot 2 Gel Transfer System (ThermoFisher). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping protein (Rockland Immunochemicals, Limerick, PA rabbit polyclonal anti-GAPDH primary antibody, 1:1000) for relative aromatase abundance calculation (ThermoFisher PA1-21398 rabbit polyclonal anti-aromatase primary antibody, 1:100). Following secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit; GE Healthcare Bio-Sciences, Pittsburgh, PA NA934, 1:10,000) and application of SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher 34577), bands were

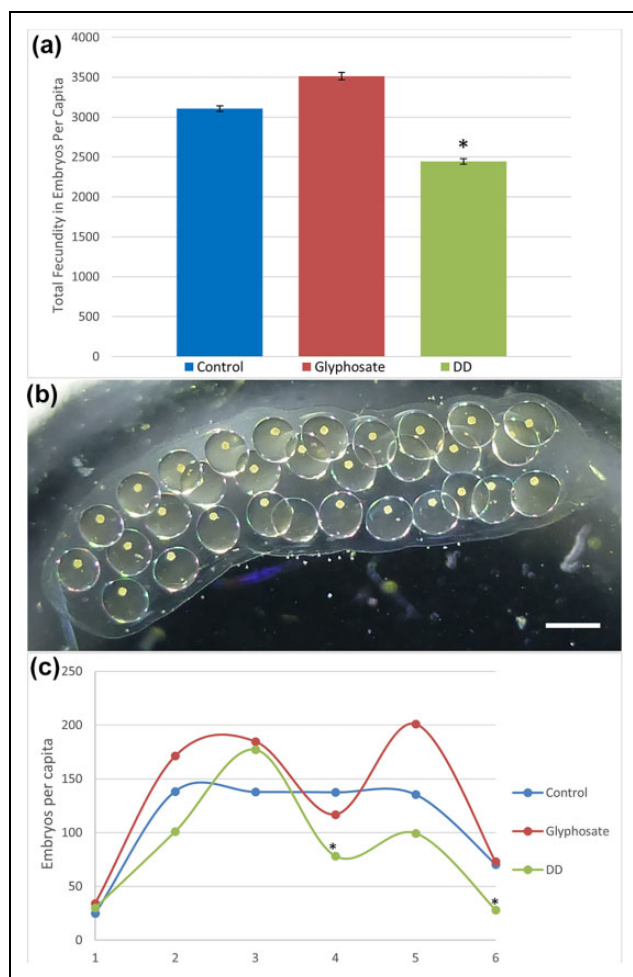


Figure 2. Fecundity of *L. palustris* following treatment with glyphosate or DD. (a) Total fecundity per snail in control APW, 3.5 mg/L glyphosate treatment, and 140 µg/L DD treatment over 3-week study. Twice weekly harvested embryos were normalized to number of living individuals to obtain a per capita count. The six per capita counts were averaged and fecundity in each treatment was compared to control fecundity using two-tailed *t*-test; **p* < 0.05. Error bars represent SEM. (b) Jelly mass of *L. palustris*; bar = 1 mm. (c) Average fecundity per capita in each treatment over the course of the study was assessed twice weekly with embryo counts per live individual numbered sequentially 1–6. Embryo counts at each time point in each treatment were compared to control embryo counts using two-tailed *t*-test; **p* < 0.05. DD: diquat dibromide; APW: artificial pond water; SEM: standard error of the mean.

imaged on a Bio-Rad ChemiDoc-MP and quantified using Image Lab software (Bio-Rad Hercules, CA).

To analyze StAR abundance in whole snail, ovotestis, kidney, or brain, tissues were isolated and disrupted similarly using ReadyPrep Protein Extraction Kit (Bio-Rad 163-2086). Samples were quantified using EZQ Protein Quantitation Kit (Invitrogen Carlsbad, CA/ThermoFisher R33200), and 30 µg of total protein or 10 µg of individual organ protein was loaded in each lane for 12% PAGE and transfer as above. Primary anti-StAR at 1:1000 and

subsequent secondary, treatment, and analysis as described above to quantify relative intensity of bands in control and treated samples.

Statistical analysis

Paired *t*-tests comparing glyphosate and DD treatment groups relative to controls were conducted on embryo counts (fecundity), hemolymph concentrations of estradiol and testosterone, and relative abundance of StAR protein present in control, glyphosate-, and DD-treated samples. Normalized abundance of aromatase protein present in control, glyphosate-, and DD-treated samples were compared by χ^2 analysis using the control levels as the expected range.

Results

Fecundity

Embryo counts collected from each mesocosm were normalized by the number of snails alive at that time point to ensure accurate representation of the fecundity per individual for each treatment type over the 3-week study (Figure 2(a)), since a low level of spontaneous mortality occurred. As the study progressed, DD-treated snails exhibited a marked decrease in embryo production (**p* < 0.05). The fecundity of glyphosate-treated snails did not differ significantly from the control animals over the course of the study, and at several time points exceeded control fecundity (Figure 2(c)). To determine the cause of the disruption in fecundity, steroid sex hormone levels and steroidogenic pathway component abundance were analyzed.

Steroid hormone levels

Hemolymph extracted from animals prior to the outset of the study and weekly for 3 weeks during treatment was analyzed by ELISA for estradiol and testosterone concentrations. In glyphosate-treated animals, we observed a consistent trend of estradiol concentration comparable to or exceeding that of control animals (Figure 3(a) and (b)). Testosterone levels in glyphosate-treated animals are statistically comparable to control animals (Figure 3(c); *p* = 0.06), although consistently lower than controls and in decreasing concentration relative to control animals as the study progressed beyond the 1-week mark (Figure 3(d)).

Hemolymph of animals treated with DD contains significantly lower estradiol (Figure 3(a) and (b)) and testosterone (Figure 3(c) and (d)) concentrations relative to control animals. The differences were most pronounced at the 3-week mark, when estradiol and testosterone concentrations in DD-treated animals are lowest relative to controls (***p* < 0.01).

Western blot analyses

Western blot analysis on isolated ovotestes from the various treatment groups at the conclusion of the 3-week study

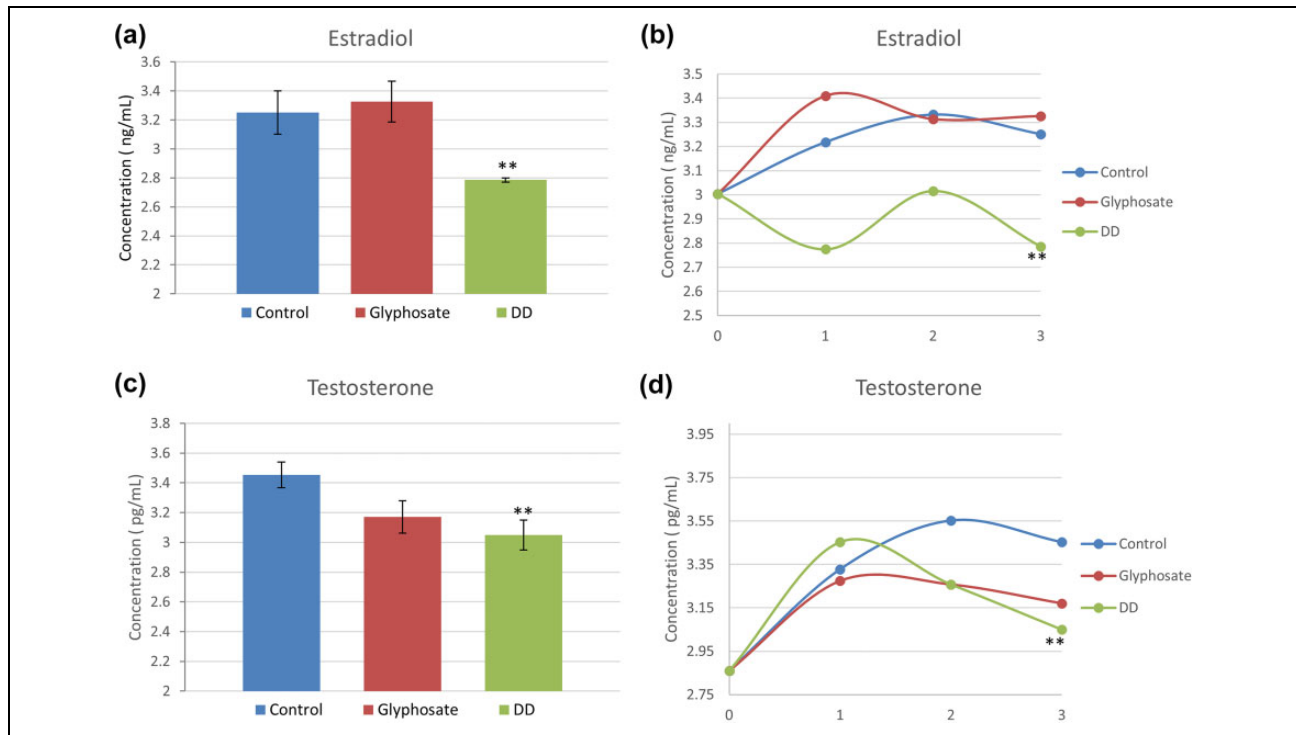


Figure 3. Testosterone and estradiol levels measured by ELISA in *L. palustris* hemolymph throughout 3-week exposure to glyphosate or DD. (a) Hemolymph estradiol concentration in ng/mL at the conclusion of the study. (b) Graph of fluctuations in estradiol concentration over course of weekly hemolymph draw and analysis; zero represents the draw prior to the outset of treatment and each number represents the draw at the conclusion of each week. (c) Hemolymph testosterone concentration in pg/mL at the conclusion of the study. (d) Graph of fluctuations in testosterone concentration over course of weekly hemolymph draw and analysis; zero represents the draw prior to the outset of treatment and each number represents the draw at the conclusion of each week. Error bars represent SEM; ** $p < 0.01$. DD: diquat dibromide; ELISA: enzyme-linked immunosorbent assay; SEM: standard error of the mean.

revealed that glyphosate-treated samples on average had a larger quantity of aromatase protein compared to control samples, and DD-treated samples had a lower quantity of aromatase protein compared to control samples. Although some DD-treated ovotestis samples had comparable aromatase or approximately two- to three-fold less than control samples, most had little to no detectable aromatase, although GAPDH detection reveals there was equivalent protein present in the sample (Figure 4(a)). Ovotestes from glyphosate-treated animals contained the highest levels of aromatase protein across all samples analyzed ($n = 44$); however, there was no significant difference in aromatase level between control and glyphosate-treated samples, and glyphosate-treated samples in particular exhibited very high variability between individuals. When analyzed in sets corresponding to separate Western blots (each set containing DD-treated, glyphosate-treated, and control individuals' ovotestis), the distribution of aromatase content in DD and glyphosate samples was found to differ greatly ($p < 0.01$) from the normal range of distribution found in control samples (Figure 4(b)).

StAR protein is readily detected in snail brain, kidney, and ovotestes, but is not detected in other snail organs tested (lung, heart). Following 6-week chronic treatment

in Roundup, DD, or glyphosate, whole snails as well as isolated kidney, brain, and ovotestes were analyzed to quantify decrease in StAR expression (Figure 5). Glyphosate-treated whole animal samples exhibited a 42% reduction in StAR, Roundup-treated samples on average exhibited a 65% reduction in StAR, and DD-treated whole animal samples exhibited a 70% reduction in StAR (Figure 5(a) and (b)), suggesting that both active ingredients of Roundup may contribute to the downregulation of StAR in the whole animal when chronically exposed. When individual organs from chronically treated animals were analyzed, the organs were affected differentially by the same treatments (Figure 5(c)). The kidney exhibited a less than two-fold reduction in StAR following 6-week treatment with Roundup (62% of control) or DD (55% of control), while the brain exhibited greater than four-fold reduction in StAR following 6-week treatment with Roundup (22% of control) or DD (23% of control), and the ovotestis was the steroidogenic organ most affected by Roundup (6% of control) or DD (9% of control) with greater than 13-fold reduction in StAR.

A summary of the individual effects of glyphosate and DD in our system is found in Table 1. While glyphosate is associated with a transient rise in fecundity, a trend toward

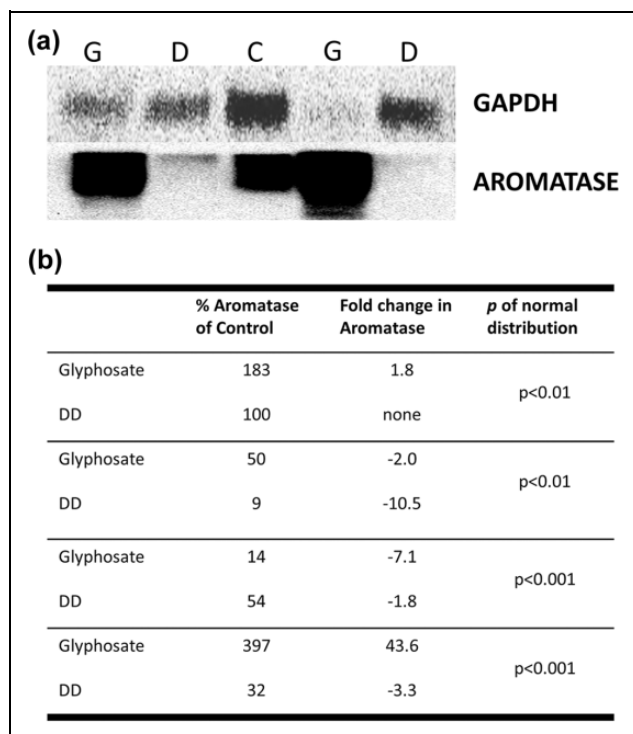


Figure 4. (a) Representative Western blot of ovotestes harvested from animals exposed to 3-week treatment in control APW, glyphosate (3.5 mg/L), or DD (140 µg/L). Top: Western blot of GAPDH for internal standardization of samples. Bottom: Western blot of aromatase (G: glyphosate; D: DD; C: control APW). (b) Percent aromatase quantity in treated ovotestes relative to aromatase quantity in control ovotestes for four Western blots, demonstrating high variability of aromatase quantity in glyphosate-treated samples and consistent though variable decrease in aromatase quantity in DD-treated samples. Aromatase quantity in treated ovotestes relative to aromatase quantity in control ovotestes is also represented as fold change in aromatase. χ^2 analysis conducted on each individual blot comparing average quantity of aromatase in each treatment group relative to average quantity of aromatase in control group; treatment group aromatase levels varied significantly from expected normal distribution based on control group for each blot. APW: artificial pond water; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DD: diquat dibromide.

decreased testosterone and increased estradiol and fecundity leading to a modest increase in estradiol to testosterone (E:T) ratio, and potential elevation of aromatase level, the main impact of 3-week glyphosate treatment is a reduction in StAR level. In contrast, the effects of DD treatment include significant decreases in StAR, fecundity, testosterone, and estradiol leading to a modest reduction in E:T ratio, and a trend toward lower aromatase levels.

Discussion

Fecundity of *L. palustris* is disrupted by chronic and ecologically-relevant exposure to constituents of Roundup, with the greatest reduction observed with DD exposure,

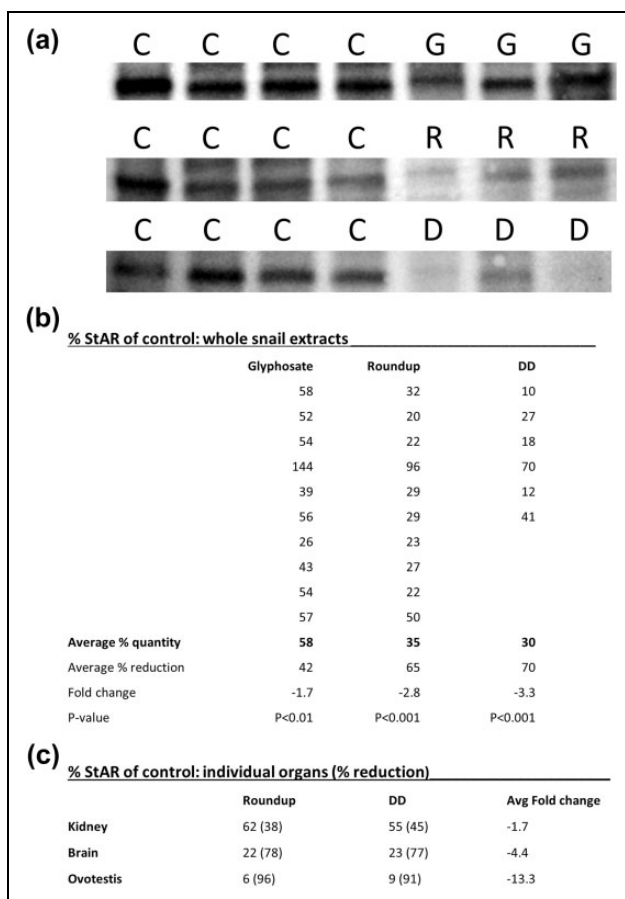


Figure 5. (a) Representative Western blots for StAR of whole snails exposed for 6 weeks to control (APW) conditions or supplemented with Roundup (19.5 mg/L), glyphosate (3.5 mg/L), or DD (140 µg/L). Representative samples from 12 control snails, 3 glyphosate-, 3 Roundup-, and 3 DD-treated snails are shown (30 µg protein total loaded per lane). C: control; G: glyphosate; R: Roundup; D: diquat dibromide. (b) Percent StAR quantity in treated whole snail extracts relative to StAR quantity in control whole snail extracts; all treated individuals except one in glyphosate exhibited a reduction in StAR, for an average reduction in StAR quantity of 41.7% for glyphosate, 65.0% for Roundup, and 70.3% for DD; average fold change in StAR in each treatment group is also shown. Each treatment resulted in significant reduction in StAR protein compared to control whole snail StAR content by *t*-test. (c) Percent StAR protein in individual organs harvested from treated snails relative to organs harvested from control snails. StAR was reduced by >90% in the ovotestis over the course of chronic DD or Roundup treatment; StAR was reduced by 77–78% in the brain and 38–45% in the kidney under the same conditions. Average fold change in StAR for Roundup and DD treatment is shown for each organ. StAR: steroid acute regulatory protein; APW: artificial pond water; DD: diquat dibromide.

and fluctuating increases being observed upon glyphosate exposure. Others have demonstrated that Roundup and glyphosate have decreased egg production, but not fertilization, in zebrafish while also altering the expression of the mRNA for different steroidogenic enzymes in males and females of this species.³⁶ We found the quantity of StAR

Table 1. Trends in reproductive indicators exhibited overall in snails exposed to glyphosate or DD relative to control snails.

	Glyphosate	DD
Fecundity	↑	↓ ^a
Testosterone	↓	↓ ^a
Estradiol	↑	↓ ^a
E:T ^b	1.03	0.93
Aromatase	↑	↓
StAR	↓ ^a	↓ ^a

StAR: steroid acute regulatory protein; DD: diquat dibromide; E:T: estradiol to testosterone ratio.

^a $p < 0.05$.

^bThe control E:T value is 0.95.

protein to be reduced in snails chronically treated with Roundup and its active components, suggesting that a decrease in the production of sex hormones could be a cause of the observed alterations in fecundity. We endeavored to determine whether changes in fecundity were mirrored by the steroidogenic hormones that influence gametogenesis and reproduction, and found that chronic exposure of the snails to DD decreased circulating concentrations of both testosterone and estradiol. In response to treatment with glyphosate, there was a tendency ($p = 0.06$) for testosterone to be decreased while circulating concentrations of estradiol were not changed.

Given the differences in concentrations of circulating gonadal steroids, we additionally sought to analyze whether the abundance of aromatase was a potential target of DD and/or glyphosate, since aromatase is directly responsible for the conversion of testosterone to estradiol. Studies by Walsh et al.¹ demonstrated that Roundup decreases the expression of StAR and the ability of a cyclic adenosine monophosphate analog (dibutyl cAMP) to induce steroidogenesis in cultured MA10 cells, while Richard et al.³⁷ observed that low doses of Roundup and glyphosate both decreased aromatase activity and Roundup decreased the amount of aromatase mRNA present in cultured placental cells. A recent study by Uren Webster et al.³⁶ reported that a low dose of Roundup decreased expression of testicular steroidogenic enzymes in zebrafish and increased expression of these enzymes in the ovary. Given that hermaphrodites possess both ovaries and testes, the findings of these research groups suggest a similar effect could contribute to suppressed steroidogenesis that subsequently decreases gametogenesis and reduces fertility and fecundity seen in *L. palustris* in our studies.

It is accepted that Roundup and its components are not alone in altering reproduction as there are a plethora of herbicides and pesticides that have been identified as endocrine disruptors³⁸ and many of these endocrine disruptors are known to impact gonadal steroidogenesis in aquatic and terrestrial species. For example, another dipyrindyl compound, paraquat, has been demonstrated to exert reproductive disturbances³⁹ in freshwater snails. In addition,

exposure to atrazine, one of the most commonly used pesticides, has been shown to produce feminization in *Xenopus laevis* and shift the steroidogenic profile of the feminized males. In feminized males, gonadal expression of aromatase was increased, leading to a decrease in testosterone and an increase in estrogen.⁴⁰ The exact mechanism by which each of the reproductive disruptors acts is not fully known and a recent study by Bouétard et al.⁴¹ demonstrated that in *L. stagnalis* exposure to diquat resulted in altered expression of more than 400 putative genes, suggesting that the effects of these types of chemicals are broad and may be interrelated. Others have worked to establish a link between glyphosate and components of the steroidogenic pathway.⁴² A recent study by Defarge and colleagues¹⁰ demonstrated that glyphosate—at concentrations below those that produced toxicity—resulted in decreased aromatase activity in the human JEG3 cell line. The results of our study support the findings of others that Roundup and its constituents (glyphosate and DD) are endocrine disruptors and as such have the potential to impact not just the intended targets, but terrestrial and aquatic invertebrates and vertebrates in many ways.

Many elements of the steroidogenic pathway have been found in common between humans and molluscs, and numerous studies demonstrate that sex steroids are synthesized from cholesterol precursors in molluscs.^{28,43} There is evidence that elevated estradiol levels increase and extend gastropod mollusc oviposition in seasonal reproducers⁴⁴; this is consistent with our observations that higher fecundity, increased aromatase abundance, and elevated estradiol levels are present in glyphosate-treated snails. Lazzara's study⁴⁵ clearly links testosterone and estradiol levels to reproductive output in zebra mussels; endocrine disruption by tributyltin and other compounds in these bivalve molluscs led to reduced testosterone levels, an altered androgen/estrogen ratio, and reduction in fecundity.

While there are commonalities in steroidogenesis, there may be differences in enzymes and/or enzymatic activity between species. Although cytochrome P450 enzymatic activity has been widely demonstrated in invertebrates including molluscs, aromatase²⁷ or aromatase-like⁴⁶ activity specifically (CYP19/p450-AROM) has been less well characterized. This may be due to changes in this enzyme over evolutionary time, and the resulting difficulty in identifying an invertebrate homologue to this and other steroidogenic pathway elements.⁴⁷ In 1974, De Longcamp et al.⁴⁸ identified a putative gonadal steroidogenic pathway in molluscs that was similar to that in vertebrates and confirmed the presence of testosterone in gonads. A more recent review by Matthiessen and Gibbs⁴⁹ discussed the production of intersex and imposex individuals in neogastropod molluscs by addition of tributyltin which increased testosterone and that this effect could be blocked by use of an androgen agonist. Bearing these studies in mind, it will be important to further characterize steroidogenesis in

molluscs and identify the enzymes responsible for this pathway in *L. palustris* and other related species.

The level of conservation between higher vertebrates and molluscs of molecules and activities in the steroidogenic pathway is becoming ever more apparent, and points to the increasing usefulness of molluscs as indicator organisms in which we are able to not only determine that toxicological harm exists, but to elucidate the specific mechanisms involved. Further, it is becoming established that hormones such as testosterone and estradiol likely play physiological roles in mollusc reproduction, and that such steroid sex hormones act as endogenous modulators of gametogenesis in molluscs.^{50,51} It is clear that using an aquatic invertebrate, such as *L. palustris*, as a model organism may provide evidence as to the potential impacts of contaminants in ground water and drinking water. Such studies may also be extended to herbicides that are present on vegetation and are transmitted to the groundwater following precipitation or washing for commercial or personal use.

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