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# TOXICITY OF GLYPHOSATE-BASED PESTICIDES TO FOUR NORTH AMERICAN FROG SPECIES

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**Abstract**—Glyphosate-based herbicides are among the most widely used pesticides in the world. We compared the acute toxicity of the glyphosate end-use formulation Roundup Original® to four North American amphibian species (*Rana clamitans*, *R. pipiens*, *R. sylvatica*, and *Bufo americanus*) and the toxicity of glyphosate technical, the polyethoxylated tallowamine surfactant (POEA) commonly used in glyphosate-based herbicides, and five newer glyphosate formulations to *R. clamitans*. For *R. clamitans*, acute toxicity values in order of decreasing toxicity were POEA > Roundup Original > Roundup Transorb® > Glyfos AU®; no significant acute toxicity was observed with glyphosate technical material or the glyphosate formulations Roundup Biactive®, Touchdown®, or Glyfos BIO®. Comparisons between the four amphibian species showed that the toxicity of Roundup Original varied with species and developmental stage. *Rana pipiens* tadpoles chronically exposed to environmentally relevant concentrations of POEA or glyphosate formulations containing POEA showed decreased snout–vent length at metamorphosis and increased time to metamorphosis, tail damage, and gonadal abnormalities. These effects may be caused, in some part, by disruption of hormone signaling, because thyroid hormone receptor β mRNA transcript levels were elevated by exposure to formulations containing glyphosate and POEA. Taken together, the data suggest that surfactant composition must be considered in the evaluation of toxicity of glyphosate-based herbicides.

**Keywords**—Glyphosate Surfactants Amphibians Endocrine disruption Thyroid hormone–receptor gene

## INTRODUCTION

Weed-control products with glyphosate as the active ingredient are among the most widely used herbicides in the world. In addition, use of glyphosate-based herbicides is increasing because of the introduction of genetically modified, glyphosate-resistant crop plants. Roundup® (Monsanto, Saint Louis, MO, USA) is a popular glyphosate-based herbicide widely used in domestic and agricultural weed control. The same formulation (under the trade name Vision®; Monsanto Canada, Winnipeg, MA, Canada) is used for site preparation and conifer release in forestry.

A number of studies have examined the toxicity of the Roundup herbicide to nontarget organisms (for a recent review, see [1]). Much of the available information reveals that end-use (formulated) glyphosate products are more toxic to aquatic organisms than the glyphosate acid active ingredient alone and that this results from the toxicity of the surfactant used in the formulation [1–5]. It has also been shown that the toxicity of the surfactant varies with temperature, pH, species, and stage of the animal exposed [2–4]. In addition to differences in acute toxicity [4], several nonionic surfactants (i.e., nonyl- and octylphenols) may act as endocrine-disrupting compounds (EDCs) and have shown estrogenicity in EDC assays [6].

With the removal of patent protection for glyphosate, which occurred in 2000, many new glyphosate-based herbicides have entered the market. Each of these products has a slightly different chemistry and surfactant mixture, and in comparison with the original Roundup formulation (Roundup Original®), some of these new products are less acutely toxic to nontarget organisms. In a recent study with Australian amphibians, the glyphosate formulations Touchdown® (Syngenta, Wilmington, DE, USA) and Roundup Biactive® (Monsanto) were less acutely toxic to larval-stage amphibians than the original Roundup formulation [4]. It was concluded that the Roundup Biactive formulation was the only herbicide tested that could be used within the margin of safety required for the protection of aquatic life in Australia and, at that time, was therefore the only formulation registered for aquatic weed control. With the exception of that study, however, there appears to be very little research concerning the environmental persistence and chronic toxicity of different glyphosate formulations and surfactant mixtures on nontarget organisms. One of the reasons is that information regarding the surfactant components of herbicide formulations is often protected as proprietary information of the manufacturer, which makes examination of individual herbicide products difficult.

Amphibian species are appropriate for examining the acute and chronic toxicity of various glyphosate-based formulations in the aquatic environment and for detecting the endocrine-disrupting potential of these herbicides. First, their dependence on aquatic sites for reproduction and early development may make amphibians susceptible to the toxic effects of contaminants such as pesticides, because their breeding sites in shallow forest and agricultural ponds can contain higher chemical contaminant levels than larger water bodies. For example, the highest environmental concentrations of glyphosate have been found in a pond environment. These ranged from 0.09 to 1.7

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mg glyphosate acid equivalents (a.e.)/L in pond water and 0.26 to 19 mg a.e./L in pond sediment [1]. Second, glyphosate may be more persistent in lentic water bodies because of less dilution and dispersion [1]. Glyphosate has an aquatic half-life ranging from 2 to 14 d, whereas that of the associated polyethoxylated tallowamine surfactant (POEA), (Monsanto's MON 0818), in the environment has been conservatively estimated at 21 to 42 d [1]. Third, because of their typical aquatic larval development and hormonally dependent metamorphosis and sexual differentiation, amphibians represent an ideal model for detecting the effects of EDCs [7–9].

Although research has been conducted regarding the acute toxicity of Roundup to amphibians [4,5], we could find no information concerning long-term exposures that encompassed the various potentially sensitive stages of amphibian development. In the present study, we compared the acute toxicity to and developmental effects on amphibians of the active-ingredient glyphosate, the POEA surfactant (using MON 0818 surfactant), and six glyphosate-based formulations. We examined differences in species and developmental stage sensitivity in acute toxicity tests with four North American amphibian species: The green frog (*Rana clamitans*), the northern leopard frog (*R. pipiens*), the wood frog (*R. sylvatica*), and the American toad (*Bufo americanus*). The formulated glyphosate-based herbicides used in the present study were Roundup Original, Roundup Transorb®, and Roundup Biactive (all from Monsanto), Touchdown (Syngenta), and Glyfos AU® and Glyfos BIO® (both from Cheminova, Wayne, NJ, USA).

We also compared the chronic effects of environmentally relevant concentrations of the various herbicide formulations on the development of Northern leopard frogs (*R. pipiens*), a widespread North American species, by examining changes in the rates of growth and development as well as by recording any malformations that occurred during development. Disruption of the thyroid axis was assessed by measuring typical physical endpoints at metamorphosis (length and rate of development) as well as by analyzing thyroid hormone receptor (TR) β gene expression. We also assessed gonadal differentiation at metamorphic climax. Amphibian sexual development (and, in particular, gonadal differentiation) is susceptible to alteration by chemical contaminant exposure [8,10,11]. The aim of the present study was to assess the acute and chronic toxicity of several glyphosate-based formulations and to determine if certain formulations pose a lower risk to the growth and development of North American amphibians. Based on related work [4], we hypothesized that our study animals would respond differently to glyphosate-based herbicides with different formulations.

## MATERIALS AND METHODS

### *Animal collection and husbandry*

The present report includes early range-finding studies with four different frog species in 1994 and more in-depth studies completed with *R. clamitans* and *R. pipiens* during the breeding seasons of 2000 and 2001. All experiments were carried out in a climate-controlled room at Trent University (Peterborough, ON, Canada), and care and treatment of the animals were in accordance with the guidelines of the Trent University Animal Care Committee. Two egg broods for each of the four amphibian species (*R. clamitans*, *R. sylvatica*, *R. pipiens*, and *B. americanus*) were collected from ponds along the Otonabee River (Canada) watershed within 5 km of Trent University (44°21′N, 78°17′W). Eggs were collected within 24 h of being laid and immediately transferred to the laboratory, where they were kept under standard conditions as previously described [12]. Once eggs of a particular species hatched, the tadpoles were randomly combined and reared in glass aquaria filled with aerated, sand-filtered water collected locally from the Otonabee River. On commencement of feeding, animals were fed cooked lettuce (1994 study) or spinach (2000 and 2001 studies) ad libitum. Food was replaced daily, and uneaten material was removed. The animals were maintained in an environmental chamber on a 12:12-h light:dark cycle. Rearing temperatures were selected to simulate pond temperatures appropriate for each species: Temperatures for the acute toxicity studies using *R. sylvatica*, *B. americanus*, and *R. pipiens* (1994 study) were 15 ± 1°C, whereas a temperature of 20 ± 1°C was maintained for the later-breeding *R. clamitans* (1994 and 2001 study). An average temperature of 20 ± 1°C was maintained for the chronic toxicity tests with *R. pipiens* in 2000. Tadpoles were staged according to the method described by Gosner [13].

### *Water quality*

Published water chemistry data regarding the Otonabee River water used for tadpole rearing shows the following parameters: pH 7.8; dissolved organic carbon, 11.6 mg/L; $Na^+$, 2.99 mg/L; $Ca^{2+}$, 38 mg/L; $Cl^-$, 3.50 mg/L; metal levels typically well below 0.03 mg/L; and no contamination with organic pollutants [10,12,14]. Basic water-quality measurements were conducted using an Oakton Waterproof pH Tester No. 2 (Oakton Instruments, Vernon Hills, IL, USA). Dissolved oxygen was measured with a YSI Model 58 (YSI Environmental, Yellow Springs, OH, USA) to the nearest 0.1 mg/L. Ammonia levels were monitored using a Hagen Ammonia Aquarium Test for Fresh Water A-7820 (Rolf C. Hagen, Montreal, QC, Canada). During animal exposures, dissolved oxygen was always at acceptable levels, and pH varied from 7.8 to 8.3. Total ammonia concentrations reached a maximum of 2.4 mg/L. All values fell within accepted guidelines [15].

### *Chemical compounds*

Six glyphosate-based formulations, the active ingredient glyphosate isopropylamine salt, and POEA used in the Roundup Original and Roundup Transorb formulations were investigated (Table 1). Technical-grade glyphosate isopropylamine salt and the POEA surfactant (MON 0818) are the main components of Monsanto's Roundup Original agricultural herbicide and Vision forestry herbicide. Roundup Transorb is applied after the addition of a commercial surfactant; the Roundup Transorb used in the present study contains a ''surfactant blend containing POEA'' (P. Marshall, Monsanto, personal communication). Roundup Biactive contains 10 to 20% of an unspecified surfactant.

Other glyphosate-based herbicides include the Glyfos products from Cheminova. Similar to Roundup, Glyfos herbicides incorporate the isopropylamine salt of glyphosate and ethoxylated tallowamines as surfactants. The formulations used in the present experiments were coded Glyfos AU herbicide and Glyfos BIO. Finally, Touchdown 480, from Syngenta/Zeneca, is based on glyphosate trimesium (the trimethylsulfonium salt) and is typically mixed with one of a number of commercial surfactants before application.

Stock solutions of each compound were prepared using deionized water just before addition to exposure water. We list concentrations of the compounds used (mg/L) as well as their

Table 1. Summary information regarding glyphosate-based herbicide formulations, the active ingredient glyphosate, and the polyethoxylated tallow amine surfactant (POEA) used in amphibian exposures

| Chemical name | Manufacturer (manufacturer code) | North American registration number | Glyphosate acid (g/L) | Surfactant (approximate amount used in formulation) |
|---|---|---|---|---|
| Technical-grade glyphosate[a] | Monsanto (MON 0139) | U.S. EPA[b]: 524-333<br>PCP[c]: 19535 | 570 | None |
| POEA[a] | Monsanto (MON 0818) | N/A[d] | N/A | Polyethoxylated tallow amines (69–73%) |
| Roundup Original®[e] | Monsanto (MON 78078) | PCP: 13644 | 360 | POEA (≈15%) |
| Roundup Transorb®[e] | Monsanto (unknown) | PCP: 25344 | 360 | Blend (≈15%) with POEA |
| Roundup Biactive®[a] | Monsanto (MON 77920) | N/R[f] | 360 | Unknown (≈10–20%) |
| Glyfos AU® herbicide[a] | Cheminova (unknown) | U.S. EPA: 4787-36<br>PCP: 24359 | 360 | Surfactant blend with 3 to 7% tallow alkylamine ethoxylate by weight |
| Glyfos BIO®[a] | Cheminova (CHA 4521) | N/R | 360 | Unknown (≈10–20%) |
| Touchdown® 480 herbicide[a] | Syngenta (YF10251) | PCP: 23971 | ≈360 | Unknown (≈10–20%) |

[a] Supplied by the manufacturer (Monsanto, St. Louis, MI, USA; Cheminova, Wayne, NJ, USA; Syngenta, Wilmington, DE, USA).
[b] U.S. Environmental Protection Agency.
[c] Pest Control Product Registration Number (Canada).
[d] Not available.
[e] Purchased from agricultural supplies retailer.
[f] Not registered in North America.

formulation glyphosate acid equivalents (FAE), which were calculated as follows: The surfactant does not contain glyphosate acid, so the FAE used for the surfactant refers to the calculated amount of glyphosate acid in its formulation equivalent, assuming the surfactant component to be approximately 15%. To calculate the FAE in each glyphosate herbicide formulation, we followed the values published by Giesy et al. [1]. Thus, for a glyphosate-based formulation of 1.0, the FAE is 0.31, and the surfactant is 0.15. In other words, 1 mg of the formulation is assumed to contain 0.31 mg of glyphosate acid equivalent and approximately 0.15 mg of POEA. The exposure concentrations were then expressed as FAE/L for each formulation.

Water samples were analyzed for residues of glyphosate (*N*-(phosphonomethyl)glycine) and its primary metabolite, aminomethyl phosphonic acid (AMPA), at the Canadian Forest Service's Great Lakes Forestry Center (Sault St. Marie, ON, Canada). In 1994, water samples were analyzed using high-performance liquid chromatography and visible wavelength detection with a Varian 5560 system (Varian Instruments, Walnut Creek, CA, USA). Water from the 2000 and 2001 experiments was analyzed using gas chromatography with nitrogen-phosphorous detection. Gas chromatography was performed on an HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA, USA) with approximate limits of quantification of 0.05 mg/L; quantitation was against an internal standard, glufosinate ammonium. Five quality-control samples from the 2000 chronic exposures showed 88% recovery of glyphosate and AMPA with high precision (5–8% coefficient of variation). The control river-water samples contained no glyphosate or AMPA levels above the limits of quantification. Very little loss of the parent compound (~2%) occurred between 2 and 94 h during the exposures, and an average of 0.05 mg/L of AMPA was found in samples collected throughout the experiment. However, because measured residue values were lower than nominal concentrations, median lethal concentration (LC50) values were calculated based on measured concentrations. Analyses of the water collected during the 1994 exposures showed similar recovery of glyphosate and AMPA with high precision (~2% coefficient of variation). The AMPA was not detected in any samples. Measured residue values 2 h after addition of the compound to the water showed close similarity to nominal values; therefore, LC50 values were calculated based on nominal concentrations.

### *Acute toxicity*

*Formulation comparison.* A toxicity comparison between the different glyphosate formulations was completed in 2001. Twenty randomly selected *R. clamitans* tadpoles at Gosner stage 25 [13] were placed in 1-L glass beakers filled with filtered river water. Because stage 25 tadpoles have commenced feeding, a small amount of cooked spinach was added to the beakers. Each batch of tadpoles was exposed to one of the test compounds listed for *R. clamitans* in Table 2. A minimum of four exposure concentrations were investigated to determine the concentration of each test compound that produced approximately 50% mortality; however, 18 mg FAE/L was the highest concentration used to maintain environmental relevance of the results. Three replicates were used per concentration, and tests were static exposures with a duration of 96 h.

*Species and stage comparison.* Comparison of species and developmental stage sensitivity to the glyphosate formulation Roundup Original was performed in 1994. We exposed tadpoles of *B. americanus*, *R. clamitans*, *R. pipiens*, and *R. sylvatica* at two life stages: Gosner stages 20 and 25. Exposures were conducted in 1-L glass beakers half-filled with filtered river water. For exposures conducted with feeding-stage tadpoles, cooked lettuce was added to the water. Ten tadpoles were exposed to Roundup Original for 96 h at concentrations of 12.9, 19.3, and 25.8 mg/L, with three replicates per concentration. These exposure concentrations were equivalent to 4, 6, and 8 mg FAE/L, respectively. Mortality was recorded to establish an LC50 when possible.

### *Chronic toxicity*

*Experimental methods.* Assessment of chronic exposure to environmentally relevant concentrations of the test compounds was performed with *R. pipiens* in 2000. Twenty randomly selected *R. pipiens* tadpoles at Gosner stage 25 were placed

Table 2. Acute toxicity (median lethal concentration values [LC50] with 95% confidence intervals in parentheses) obtained in 24-h and 96-h exposures of four amphibian species exposed to glyphosate-based herbicides, glyphosate technical material, and polyethoxylated tallowamine surfactant (POEA) at two life stages[a]

| Species | Gosner stage | Compound | LC50 | | | |
|---|---|---|---|---|---|---|
| | | | 24 h | | 96 h | |
| | | | mg/L | mg FAE/L | mg/L | mg FAE/L |
| *Rana pipiens*[b] | 25 | Roundup Original® | 11.9 (11.2–12.6) | 3.7 (3.5–3.9) | 9.2 (NR) | 2.9 (NR) |
| *R. pipiens*[c] | 20 | Roundup Original | >25.8 | >8 | 20.9 (19.8–21.9) | 6.5 (6.1–6.8) |
| *R. sylvatica*[c] | 25 | Roundup Original | 18.1 (16.7–19.6) | 5.6 (5.2–6.1) | 16.5 (15.7–17.4) | 5.1 (4.9–5.4) |
| *R. sylvatica*[c] | 20 | Roundup Original | >25.8 | >8 | >25.8 | >8 |
| *Bufo americanus*[c] | 25 | Roundup Original | 13.5 (NR) | 4.2 (NR) | <12.9 | <4 |
| *B. americanus*[c] | 20 | Roundup Original | >25.8 | >8 | 25.8 (NR) | 8 (NR) |
| *R. clamitans*[d] | 25 | Roundup Original | 6.6 (6.1–7.1) | 2.0 (1.9–2.2) | 6.5 (6.0–7.0) | 2.0 (1.9–2.2) |
| *R. clamitans*[c] | 20 | Roundup Original | >25.8 | >8 | 22.8 (21.2–24.5) | 7.1 (6.6–7.6) |
| *R. clamitans*[d] | 25 | Glyphosate technical | >38.9 | >17.9 | >38.9 | >17.9 |
| *R. clamitans*[d] | 25 | POEA | 1.1 (1.1–1.2) | 2.4 (2.2–2.5) | 1.1 (1.0–1.1) | 2.2 (2.1–2.4) |
| *R. clamitans*[d] | 25 | Roundup Biactive® | >57.7 | >17.9 | >57.7 | >17.9 |
| *R. clamitans*[d] | 25 | Touchdown® | >57.7 | >17.9 | >57.7 | >17.9 |
| *R. clamitans*[d] | 25 | Glyfos BIO® | >57.7 | >17.9 | >57.7 | >17.9 |
| *R. clamitans*[d] | 25 | Glyfos AU® | 29.1 (28.1–30.2) | 9.0 (8.7–9.4) | 28.6 (27.6–29.6) | 8.9 (8.6–9.2) |
| *R. clamitans*[d] | 25 | Roundup Transorb® | 7.4 (6.9–7.9) | 2.3 (2.2–2.4) | 7.2 (6.8–7.7) | 2.2 (2.1–2.4) |

[a] Roundup Original, Roundup Biactive, and Roundup Transorb from Monsanto (St. Louis, MO, USA); Touchdown from Syngenta (Wilmington, DE, USA); Glyfos BIO and Glyfos AU from Cheminova (Wayne, NJ, USA). FAE = formulation glyphosate acid equivalents; NR = 95% confidence intervals not reliable.
[b] 2000 Chronic study.
[c] 1994 Study.
[d] 2001 Study.

in five-gallon aquaria filled with filtered river water. Tadpole density was maintained at 350 ml of water per tadpole, and animals were cultured until they reached metamorphic climax (Gosner stage 42, indicated by forelimb emergence). Treatment aquaria were spiked with compounds listed in Table 1 once a week in a static renewal system for a total of 42 d of exposure, followed by rearing in clean water. The exposure duration was chosen based on the conservative half-life value estimated for the POEA surfactant in Roundup [1]. Exposures of 0.6 and 1.8 mg FAE/L were chosen based on the average and maximum concentrations found in the environment [1]. A half-change of water after 96 h was included to maintain low ammonia concentrations. Tadpoles that had hatched from the same two egg masses as the treatment tadpoles were maintained under similar growth conditions to serve as controls. The rate of development was assessed by recording the number of days taken to reach Gosner stage 42 (forelimb emergence) for each surviving tadpole from the first day of exposure. A 38% mortality rate was found in the control group during the course of the experiment, which is a level not unexpected for long-term chronic experiments with these animals. In addition, mortality across all treatments accumulated over the experimental period and was more likely to occur after the exposure period. The experiment was terminated when 80% or more of the surviving tadpoles in each control group reached metamorphic climax (Gosner stage 42); experiments were terminated after 166 d. For tadpoles that did not reach metamorphic climax, a maximum value was assigned for days-to-metamorphosis, which was the day of test termination. Tadpoles were euthanized using an overdose of tricaine methanesulfonate (MS-222; Syndel Laboratories, Vancouver, BC, Canada), after which Gosner stages were determined and snout–vent length and total length were measured to the nearest 0.5 mm. Each metamorph was fixed and decalcified in Cal-Ex II (Fisher Scientific, Springfield, NJ, USA) for 48 h, then placed in 70% ethanol for preservation until histological processing.

*Tadpole morphometrics and tail damage.* Twenty-eight days after termination of exposure (i.e., day 70 of the experiment), slowly developing tadpoles that remained at Gosner stage 25, as well as all other surviving tadpoles, were placed in a narrow transparent container and digitally photographed. A ruler was included to provide scale. Total length, body length, tail length, and maximum tail height were recorded using SigmaScan® Pro5 software (SPSS, Chicago, IL, USA; morphological measurements follow the method described by Altig and McDiarmid [16]). Tadpole tail effects that manifested as a reduction in tail length were measured, and visible damage to the tail was also assessed. Tail damage was visible as apparent necrosis of the tail tip with varying degrees of severity; in severe cases, flexure of the tail tip, fin damage, abnormal growths on the tail tip, and/or blistering on the tail fin were noted. If any of these defects were observed, the tadpole was scored as having tail damage. The percentage of tadpoles showing damage in each treatment group was then calculated.

*Gonadal histology and determination of sex ratios.* The Cal-Ex II–preserved abdomens of *R. pipiens* metamorphs at Gosner stage 42 were prepared for histology. Tissues were dehydrated in a standard series of alcohol treatments and then embedded into paraffin. Transverse-step sections (thickness, 7 μm) were cut through the entire area of the gonad, placed on albumin-treated slides, and stained with hematoxylin and eosin. Histological examination of gonadal tissue under a compound microscope allowed each metamorph to be identified as female, male, or intersex, as described previously [8,10,17–19] (see *Results* for further description). In addition, in a preliminary attempt to quantitate the observed differences, the diameter of 10 randomly selected oocytes was measured in gonads of all animals categorized as intersex, as was five randomly selected female gonads from the control group.

Natural sex ratios of wild populations of ranid amphibians in our study area were determined in 1993, 1994, 1996, and 1999 by examining adult frogs on migration along a 9-km

section of road between ponds where egg broods were collected [20]. Secondary sex characteristics, primarily thumb pads on males, were used to determine the phenotypic sex of spring- and fall-migrating *R. pipiens* and *R. clamitans*. Frogs found dead on the road were taken into the laboratory for further dissection to establish the presence of testes or ovaries. Juvenile animals were not included in calculations of the natural sex ratio.

*Gene expression analyses.* For mRNA expression analyses, five slowly developing tadpoles that remained at Gosner stage 25 were randomly selected from each treatment at day 70 of the experiment (as described above). Five tadpoles were also randomly selected from each treatment once they reached stage 42. In preparation for total RNA isolation, the tadpole tail tips were preserved in RNAlater (Ambion, Houston, TX, USA). Total RNA from tadpole tails was isolated using TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada) and processed as described previously [21,22]. Specific TRβ and L8 ribosomal protein cDNA sequences were cloned from *R. pipiens* tadpole homogenates using primers designed against the corresponding *Xenopus laevis* cDNA sequence employing methods previously described [21]. The DNA sequences obtained were submitted to GenBank (accession nos. AY049025, AY049026, and AH011141). For normalization across samples, ribosomal protein L8 mRNA was chosen (see Crump et al. [21]). Finally, isolated total RNA (1 μg) was used to produce cDNA as described previously [21]. Steady-state levels of L8 and TRβ mRNA were analyzed using an MX4000 real-time quantitative polymerase chain reaction system (Stratagene, La Jolla, CA, USA). Amplification reactions were run as previously described [21], and all details regarding reaction mixtures and conditions are reported therein. The experiments were performed in triplicate, and the data obtained were averaged and normalized to the ribosomal L8 control.

### *Statistical analyses*

The LC50 values for 24- and 96-h exposures were calculated using available software (CEE Computer Model Library, Old Dominion University, Norfolk, VA, USA) based on a trimmed Spearman-Karber method [23,24]. Differences in species and stage were compared using the Fisher Exact chi-square test (StatSoft®, Tulsa, OK, USA), with significance being accepted at a Bonferroni adjusted $p < 0.05$. Anatomical measurements of tadpoles (total length, tail length, body length, and maximum tail height) and metamorphs (snout–vent length) and days-to-metamorphosis were analyzed using analysis of variance least-significant-difference tests (StatSoft), with significance being accepted at $p < 0.05$. Sex ratios were compared to an expected 50:50 (male:female) ratio using an observed-versus-expected chi-square test, with significance being accepted as described above. The number of tadpoles to reach metamorphic climax was compared to that of the control group using an observed-versus-expected chi-square test (StatSoft), with significance being accepted as described above. The number of treated tadpoles with observable tail damage was compared against that of controls using Fisher's exact test (SAS Institute, Cary, NC, USA). Control TRβ mRNA levels were compared with each individual treatment using a Mann–Whitney *U* test with Bonferroni correction to $p = 0.016$ for significance (SPSS Ver 11.0).

## RESULTS

### *Acute toxicity*

The eight treatments varied in their toxicity to *R. clamitans* (Table 2): POEA, Roundup Original, Roundup Transorb, and Glyfos AU all caused sufficient mortality to allow calculation of LC50 values, whereas Touchdown, Glyfos BIO, Roundup Biactive, and glyphosate technical resulted in no mortality at concentrations up to 17.9 mg FAE/L. The surfactant POEA by itself was the most toxic compound examined, and glyphosate formulations known to contain ethoxylated tallowamine surfactants (Roundup Original, Roundup Transorb, and Glyfos AU) were more toxic than other glyphosate formulations. At concentrations approaching the 96-h LC50 values for POEA, Roundup Original, Roundup Transorb, and Glyfos AU, tail damage occurred in 52 to 71% of surviving *R. pipiens* tadpoles within the first 24 h of exposure (data not shown).

Tadpoles at Gosner stages 20 and 25 showed different sensitivity to exposure. After 24 h of exposure, insufficient mortality had occurred to calculate LC50 values for stage 20 tadpoles of *R. clamitans*, *R. pipiens*, *R. sylvatica*, and *B. americanus* exposed to technical-grade glyphosate (data not shown) or Roundup Original (Table 2). However, at stage 25, all species showed sensitivity ($p < 0.005$ for all treatments against stage 20 animals) of varying degrees, with *R. clamitans* (the most sensitive) and *R. sylvatica* (consistently the most tolerant; $p < 0.01$) representing the two extremes in response.

### *Chronic toxicity*

*Metamorphosis endpoints.* The average value for days-to-metamorphosis was 120 ± 36 d, and the experiment was terminated after 166 d. The rate at which tadpoles reached metamorphic climax and the number of metamorphs present (i.e., the proportion of surviving larvae to reach Gosner stage 42) were significantly reduced following exposure to POEA, Roundup Original, and Roundup Transorb (Fig. 1a and b). Some tadpoles developed only very slowly (and were still at stage 25 at 70 d after the start of the experiment), but these were otherwise normal animals that were held back in their development, likely as a result of effects of animal density or social interactions that can lead to developmental delays in anuran tadpoles reared in the laboratory. Tadpoles exposed to POEA, Roundup Original, and Roundup Transorb were significantly smaller compared to animals exposed to glyphosate technical or the control animals (Fig. 1c).

*Histology and sex ratios.* Because previous studies regarding pesticides have shown potential effects on amphibian gonadal development [10,11,25], we examined the gonadal histology of Gosner stage 42 animals after chronic exposure to glyphosate formulations. Representative histological sections of *R. pipiens* stage 42 gonads are shown in Figure 2. Normal female ovaries (Fig. 2a) usually contained a thin germinal epithelium, many large primary oocytes (diameter, 50–199 μm), and some oocytes containing cortical alveoli showing slight variation in maturity (morphology described by Witschi 1929 [17]). Normal testes of stage 42 male metamorphs (Fig. 2b) were generally immature, containing spermatozoa surrounded by follicle cells with undefined seminiferous tubules [17]. We observed metamorphs with abnormal gonads after exposures to both concentrations of POEA, Roundup Original, and Roundup Transorb (Figs. 2 and 3). Individuals were categorized as intersex when we observed maturing primary oocytes (diameter, 50–161 μm; with at least one oocyte 100 μm

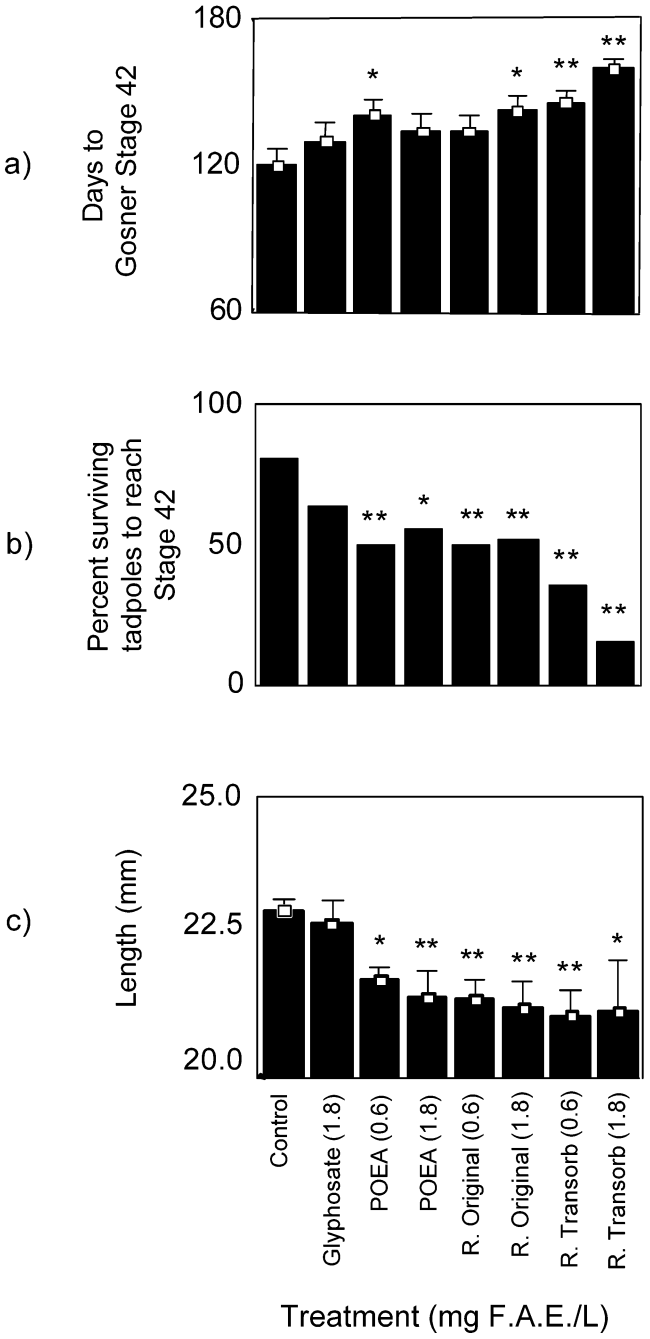


Fig. 1. Metamorphosis data for *Rana pipiens* tadpoles exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA) and Roundup Transorb® (R. Transorb; Monsanto). **a.** Days-to-metamorphic climax. **b.** Percentage of surviving tadpoles to reach metamorphic climax. **c.** Snout–vent length (mm). Compound concentrations in mg formulation acid equivalents (FAE; see text)/L follow compound names. Bar height represents the mean, and error bars represent the standard error. $*p < 0.05$, $**p < 0.01$ compared to control.

in diameter in each gonad), sometimes containing cortical alveoli, surrounded by varying degrees of somatic and/or medullary tissue (Fig. 2d). The degree of proliferation of medullary or somatic tissue varied from an abnormal lining of an ovarian cavity to completely intersex gonads with ovarian tissue at the posterior end and testicular tissue at the anterior end (similar to the findings of previous studies [8,17]). The other form of intersex observed included an enlarged germinal epithelium with a proliferation of oogonia and atretic oocytes (Fig. 2c, as also observed previously [19]); this was found in metamorphs exposed to Roundup Original and POEA. The observed sex ratio of control animals is comparable to that found in the local, natural population of adult *R. pipiens* and *R. clamitans* (52:48, female:male; $n = 794$; M. Berrill, unpublished data). Although intersex animals were observed after exposures to POEA, Roundup Original, and Roundup Transorb (glyphosate alone had no effect), none of the sex ratios from any of the treatment groups was significantly different from an equal sex ratio (Fig. 3).

*Tail damage.* Exposure to the POEA surfactant or the glyphosate formulations Roundup Original and Roundup Transorb resulted in an increased time to metamorphosis in many cases. At the same time, exposure to these compounds caused an increased frequency of tail damage (Fig. 4a). Tail damage seemed to occur through necrosis of the tail tip and, in severe cases, was characterized by flexure of the tail tip, fin damage, abnormal growths on the tail tip, and/or blistering on the tail fin (e.g., see Fig. 4f). Tail damage was not observed in tadpoles exposed to glyphosate alone, whereas the frequency of tail damage in tadpoles exposed to high concentrations of POEA, Roundup Original, and Roundup Transorb reached 94% (Fig. 4a). The tail damage persisted postexposure until metamorphic climax in surviving animals. This is associated with decreased tail length (Fig. 4b), whereas significant differences were not seen with other tadpole measurements, such as maximum tail height (control maximum tail height, $1.09 \pm 0.25$ cm) and body length (control body length, $1.71 \pm 0.33$ cm).

*Gene expression analyses.* Tail regression is an important hallmark of metamorphosis and requires thyroid hormone–dependent changes in gene expression. The genetic program is regulated by thyroid hormone–specific nuclear transcription factors, TRα and TRβ [26]. In contrast to TRα, the expression of TRβ is highly thyroid hormone–responsive and has been used to detect perturbation in thyroid hormone signaling [21,27]. To determine whether the incidence of tail damage was associated with a change in TRβ mRNA expression, we assessed steady-state levels of TRβ mRNA in tail samples from stage 25 and stage 42 *R. pipiens* tadpoles exposed to glyphosate alone, POEA, and glyphosate formulations.

The relative copy number was approximately 10-fold higher in stage 42 tadpole tails compared to stage 25 tails (compare controls in Fig. 5). This corresponds well with the relative levels reported in *X. laevis* tadpole tail tissue [22]. Comparison of control TRβ mRNA levels in stage 25 tadpoles reveals a significant increase on exposure to the high concentration of Roundup Original and both concentrations of Roundup Transorb (Fig. 5a). In contrast, stage 42 tadpole tails showed no significant differences compared to the control (Fig. 5b).

## DISCUSSION

The present results indicate that formulations of the pesticide glyphosate that include the surfactant POEA at environmentally relevant concentrations found in ponds after field applications can be toxic to the tadpole stages of common North American amphibians. In contrast, glyphosate alone and recently developed formulations lacking POEA are less toxic. Differing effects of the different formulations can be seen in acute and chronic exposure experiments and can be measured by assessments of mortality, tadpole tail damage and length, development to metamorphosis, length at metamorphosis, TRβ

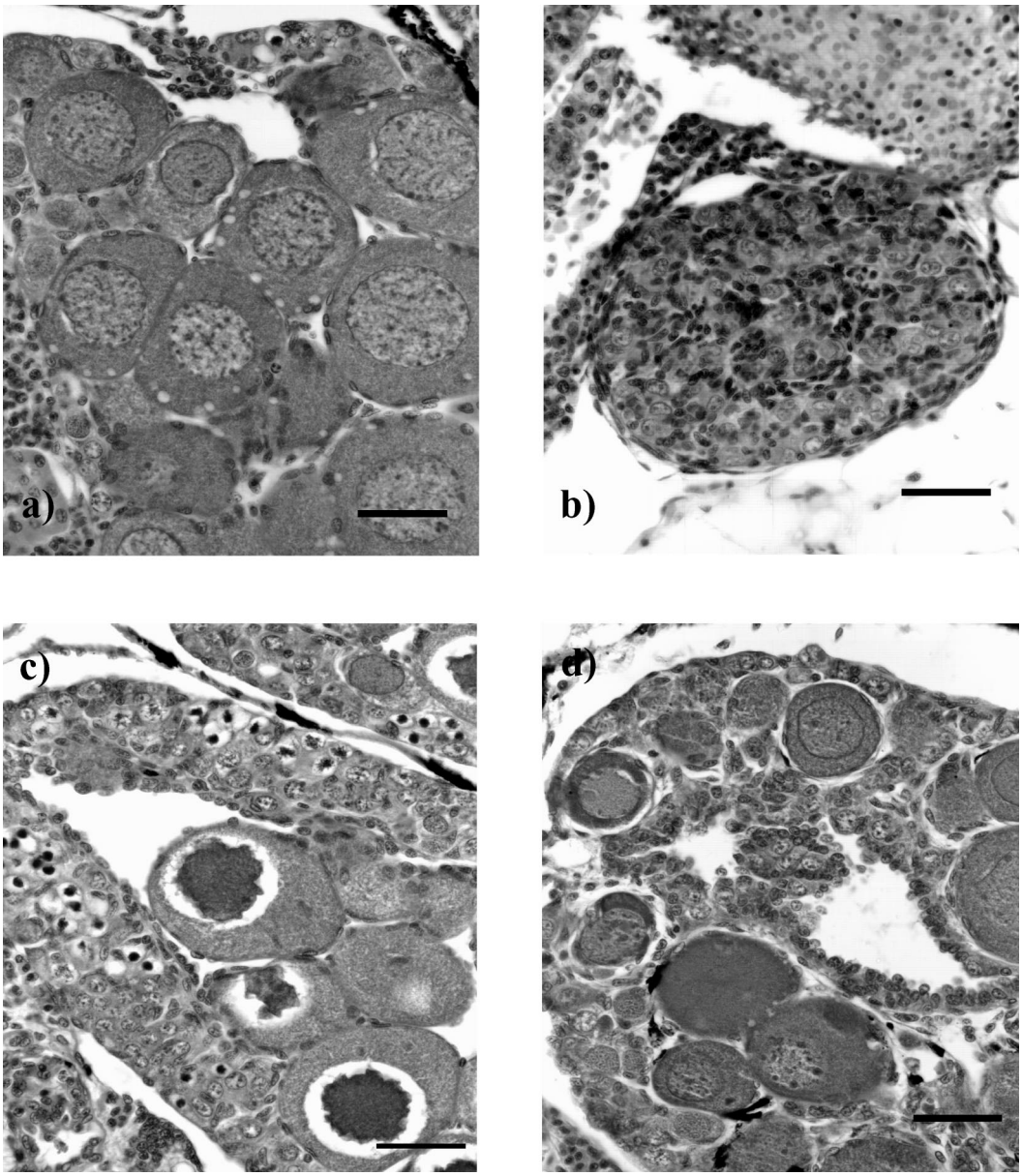


Fig. 2. *Rana pipiens* gonads at metamorphic climax (Gosner stage 42). (**a**) Control female ovary showing maturing primary oocytes with cortical alveoli. (**b**) Control male testis with spermatozoa. (**c**) Abnormal gonad showing enlarged germinal epithelium and proliferation of oogonia surrounding atretic primary oocytes (Roundup Original®; Monsanto, St. Louis, MO, USA; 1.8 mg formulated acid equivalents (FAE; see text)/L). (**d**) Abnormal gonad showing maturing oocytes containing cortical alveoli surrounded by male spermatozoa and somatic tissue (Roundup Original, 1.8 mg FAE/L). Bar = 50 μm.

mRNA expression, and occurrence of abnormal gonads. Tail reduction and presence of abnormal gonads in tadpoles exposed to the surfactant POEA and Roundup formulations suggest disruption of the thyroid axis. Previous studies have shown that thyroid hormones are involved in gonadal development (for review, see [8]).

Previous studies have also shown that Roundup herbicide formulations are moderately toxic to amphibians [1]. In the present investigation, Roundup Original was the most toxic formulation, causing the highest mortality and resulting in the lowest LC50s of all compounds tested (Table 2). This was also observed in a comparison of the toxicity of various glyphosate formulations conducted with Australian frogs [4]. On the other hand, exposure to technical-grade glyphosate, Touchdown 480, Roundup Biactive, and Glyfos BIO did not cause mortality, and Glyfos AU caused limited mortality, even at the highest concentration tested.

Exposure to POEA alone showed toxicity similar to that of the Roundup Original formulation when the toxicity data were compared on the basis of FAE concentrations. Therefore, it appears that the POEA surfactant contributes most, if not all, of the acute toxicity of Roundup Original. The acute toxicity of the surfactant may be attributed to the fact that in fish, surfactants interfere with gill morphology and cause lysis of gill epithelial cells [28], which may be followed by loss of osmotic stability and asphyxiation [29]. The toxicity of the POEA surfactant has been noted in experiments with fish and invertebrates [2,3,30] and other amphibians [4,5]. In light of these observations, it may be possible to make conclusions about the toxicity to nontarget aquatic organisms of other gly-

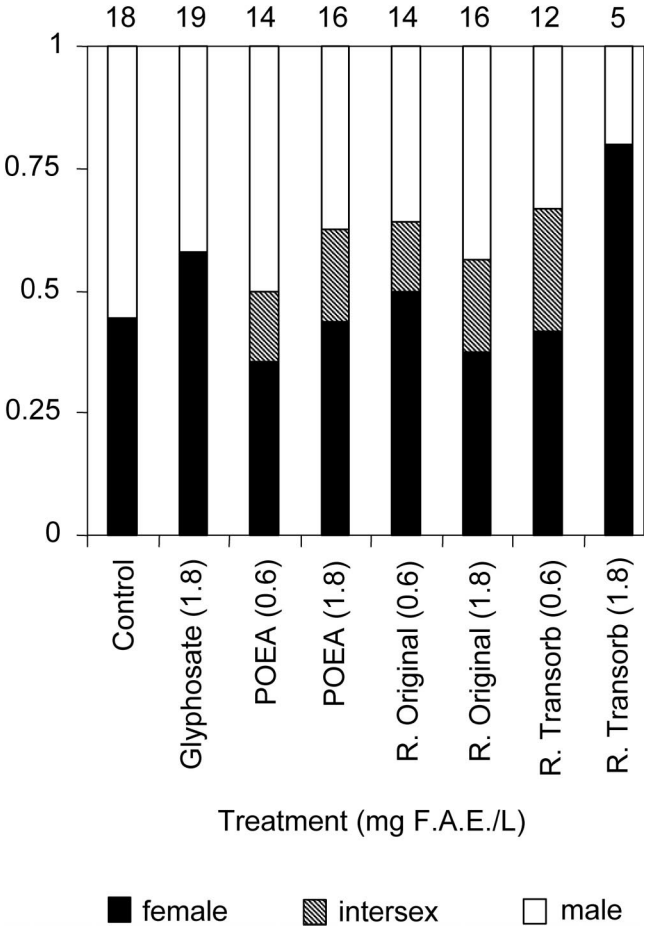


Fig. 3. Sex ratio of control and treated metamorphs based on observations of gonadal histology at metamorphic climax (Gosner stage 42). Tadpoles were exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA), and Roundup Transorb® (R. Transorb; Monsanto). Compound concentrations in mg formulation acid equivalents (FAE; see text)/L follow compound names. Numbers above bars = *n*.

phosate-based formulations containing similar surfactants. For instance, Glyfos AU, which also contains ethoxylated tallowamines as a surfactant, showed slight toxicity to amphibians. This supports the suggestion that the toxicity of glyphosate herbicide formulations is correlated with the percentage of POEA in the formulation [30].

Based on FAE, our LC50 values are higher than the residue levels that are typically measured in the environment. This suggests that residues from applications of glyphosate-based pesticides would not be acutely toxic to amphibians in the wild, but this is mainly based on information regarding the presence of glyphosate acid in the water column. Little information is available concerning the levels of POEA in ponds after field applications, and further information about the levels and environmental persistence of POEA is required for a complete assessment of the acute and chronic toxicity of these herbicide formulations. In addition, in the wild, tadpoles may be exposed to higher concentrations of these herbicides than those used in the present study, because the feeding behavior of tadpoles includes grazing on sediment and vegetation. Besides uptake by plants, glyphosate adsorbs to suspended particulate matter and sediments in water [1]. This provides another route of exposure for these animals, and these alternative routes should be taken into account for a complete assessment of the toxicity of these compounds to amphibians.

Of the species tested, *R. sylvatica* was the least sensitive and *R. clamitans* the most sensitive to herbicide exposure. In fact, *R. clamitans* appears to be among the most sensitive amphibian species examined to date, exhibiting the lowest LC50 values for amphibian species exposed to glyphosate-based pesticides reported in the literature [4,5]. In a study with the Roundup formulation that employed the Frog Embryo Teratogenesis Assay–Xenopus (FETAX) assay technique, the *X. laevis* LC50 value was 4.5-fold higher [5]. Whereas the two species, developmental stages, and exposure techniques cannot be compared, the increased sensitivity of the native species compared to the standard amphibian test species (*X. laevis*) is noteworthy. Previous experiments have shown that species vary in sensitivity depending on the contaminant tested [31], and our data support the conclusion that trends in sensitivity are not based simply on relatedness between species [12,14,32]. It has also been suggested that sensitivity to glyphosate herbicide exposure may be related to the size of the tadpole [4]. We observed differences in sensitivity between the two tadpole developmental stages examined, with new hatchlings at Gosner stage 20 being more tolerant compared to older, stage 25 tadpoles. A trend of increasing sensitivity with age in young aquatic organisms has been observed in both amphibians [12,32,33] and fish [2]. Stage 20 tadpoles may be more tolerant for a number of reasons. For instance, because they receive nourishment from a yolk sac, they do not consume potentially contaminated food sources. In addition, the sensitivity of these animals increases with organogenesis, metabolism, and immune system development [33].

In chronic exposures, glyphosate formulations containing POEA increased the incidence of tail damage and gonadal abnormalities and decreased the snout–vent length of *R. pipiens* metamorphs. Other effects included a decrease in the rate of development, a reduction in the number of animals reaching metamorphosis, and an alteration in TRβ mRNA levels. The incidence of tadpole tail damage increased with increasing exposure concentration and persisted postexposure until metamorphic climax. Treatment with the two Roundup formulations and with the POEA surfactant at 1.6 mg FAE/L resulted in tadpole tails 20 to 25% shorter than those of the control animals or tadpoles exposed to technical glyphosate alone. This loss of tail length could influence locomotory ability and increase chances of predation [34]. Contaminants have been shown to induce tail damage in wild populations of tadpoles as a result of stress [35], which suggests a mechanism that includes hormone disruption. The stress-induced hormone, corticosterone, can trigger premature tail resorption in tadpoles by enhancing the metabolism of thyroxine [36]. At the same time, strictly in terms of the nature of the tail damage we observed, this could simply be a result of damage to the thin epidermis and connective tissue making up the tail fin, as has been seen with teleost epithelial cells exposed to certain surfactants [28].

The TRβ mRNA expression is an important molecular marker of thyroid hormone–dependent metamorphosis [26]. Recent laboratory experiments have shown that the herbicide acetochlor significantly enhances the expression of TRβ mRNA in *R. catesbeiana* and *X. laevis* tail tissue [21,27]. This increase in TRβ mRNA expression correlates with accelerated precocious metamorphosis in *X. laevis* [21], which was not clearly apparent in the present study. However, we found an

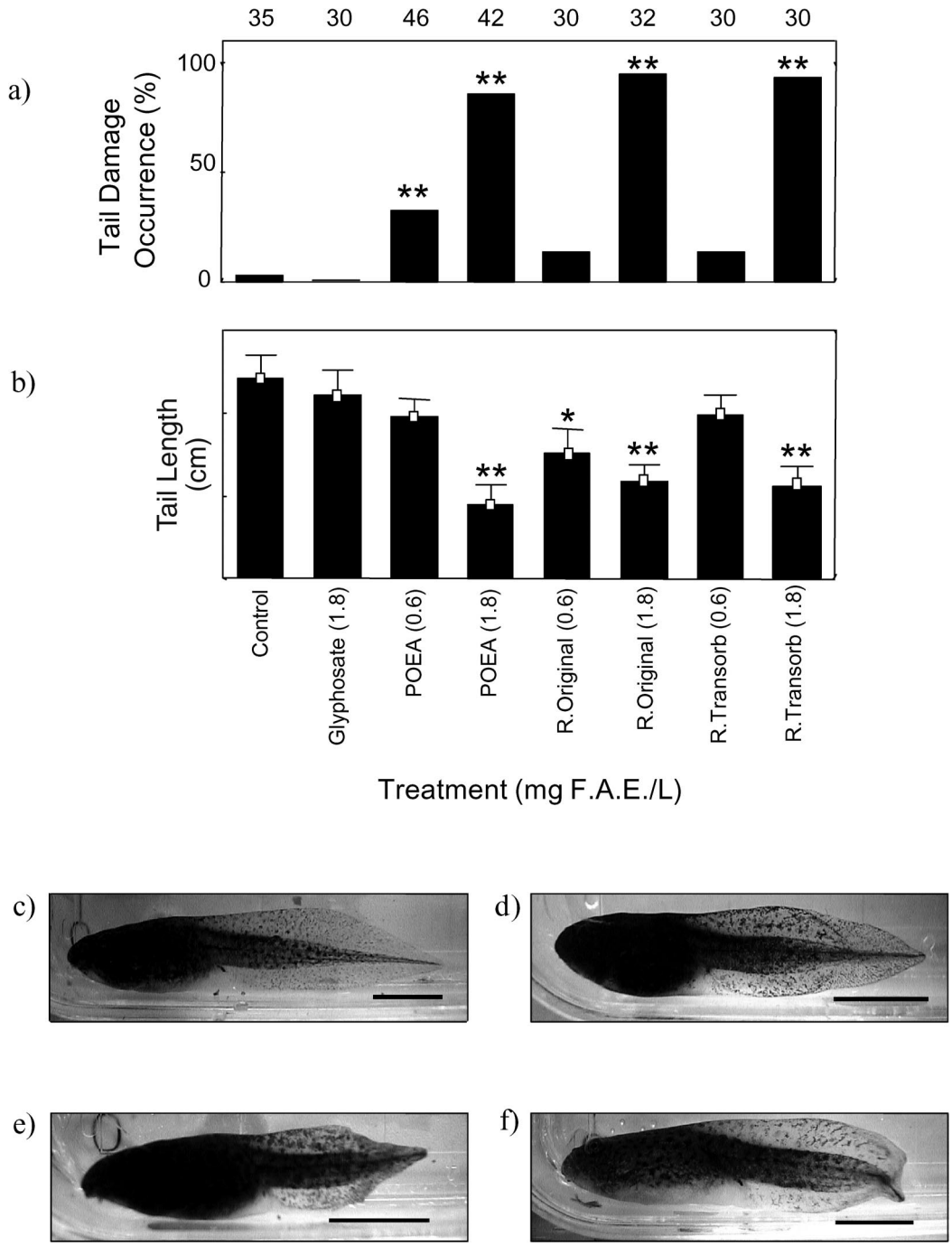


Fig. 4. Tail abnormalities observed in *Rana pipiens* tadpoles exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA), and Roundup Transorb® (R. Transorb; Monsanto). (**a**) Percentage tail damage in each treatment. (**b**) Tail length (cm). Sample size (*n*) is displayed along the top. Bar height represents the mean, whereas error bars represent the standard error. Compound concentrations in mg of formulation acid equivalents (FAE; see text)/L follow compound names. $*p < 0.05$, $**p < 0.01$. (**c**) Control tadpole showing undamaged tail fins. (**d**) Slight fin damage with necrosis (Roundup Original, 1.8 mg FAE/L). (**e**) Severe damage (Roundup Original, 1.8 mg FAE/L). (**f**) Severe fin damage plus ventral tail tip flexure (Roundup Transorb, 1.8 mg FAE/L). Bar = 1 cm.

increase in TRβ transcripts in the tails of Gosner stage 25 tadpoles on chemical treatment, which is suggestive of abnormal initiation of tail regression. In the tails from animals that survived to stage 42, we did not observe significant changes in TRβ transcript levels. The importance of altered TRβ expression levels remains to be determined, as does the longer-term implications for the animal. However, these findings are suggestive of a possible disruption of the thyroid axis by POEA, and by glyphosate formulations containing it, at a crucial time during metamorphosis that may subsequently lead to developmental abnormalities.

An effect on thyroid hormone–dependent development was also suggested by a significant decrease in the percentage of surviving tadpoles that reached stage 42 and by their delayed rate of metamorphosis after exposure to Roundup Original, Roundup Transorb, and POEA. In addition, treatment with both low and high concentrations of Roundup Original, Roundup Transorb, and POEA resulted in metamorphs that were smaller

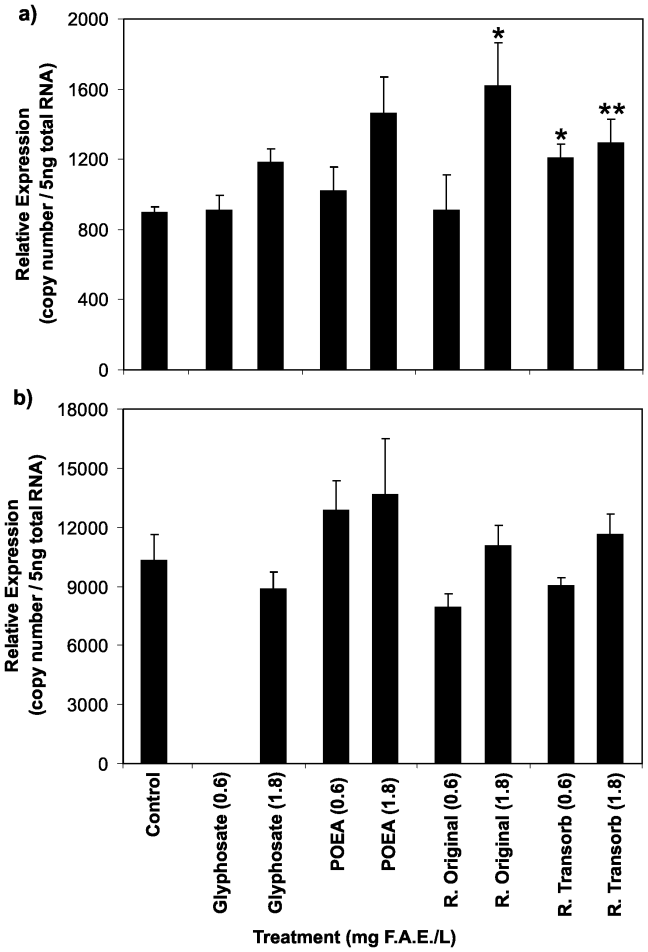


Fig. 5. Relative expression levels of thyroid hormone receptor β (TRβ) mRNA in the tails of Gosner stage 25 (**a**) and stage 42 (**b**) *Rana pipiens* tadpoles exposed to glyphosate technical, the polyethoxylated tallowamine surfactant (POEA), Roundup Original® (R. Original; Monsanto, St. Louis, MO, USA), and Roundup Transorb® (R. Transorb; Monsanto). Bar height represents the mean, whereas the error bars show the standard error. Compound concentrations in mg formulation acid equivalents (FAE; see text)/L follow compound names. $*p \leq 0.016$, $**p < 0.01$.

in size than the control metamorphs. At present, the precise mechanisms behind these effects are not known, and further study is warranted. Delay or inhibition of metamorphosis could be lethal to tadpoles developing in ephemeral ponds, and the smaller size at metamorphosis could increase the chances of predation [37] or diminish later fitness [33]. Although shorter length has been correlated with tail damage in the past [34], it does not explain the decrease in length of the tadpoles that did not experience tail damage in the present study, suggesting that other factors were also involved.

Exogenous compounds can influence development and differentiation of amphibian gonads [8]. This can result in intersex gonads and skewed sex ratios of amphibian populations at metamorphosis [10,11]. Sex ratios between treatments were not significantly different. However, intersex animals were observed after exposures to both concentrations of Roundup Original and POEA and to the lower concentration of Roundup Transorb and a preponderance of female animals at the higher Transorb concentration (although sample size was small; see Fig. 3). The forms of intersex observed have been recorded previously and are induced by abnormal environmental temperatures or by EDCs [8,17,19,38,39]. Because animals in the present study were maintained at environmentally appropriate temperatures, other contributing factors played a role in the abnormal gonadal development. Sex-steroid levels are controlled through the central nervous system, hypothalamus, anterior pituitary, and peripheral gonads themselves; chemical-induced disruption in any these areas could influence estrogen levels in the developing tadpole [40] and influence gonadal development. Past studies have shown that changes in thyroid hormone levels do not affect gonadal development directly [19,41,42]; however, sex steroids have been suspected of altering sexual characteristics by interfering with the thyroid axis [8]. In addition, thyroid hormones potentiate estrogen action and the transcription of the estrogen-receptor gene in amphibians, initiating at metamorphic climax [43]. The exact mechanism of the abnormal sexual development induced by Roundup Original, Roundup Transorb, and POEA is unclear, but it is interesting to note that Roundup can inhibit steroidogenesis by interfering with the transport of cholesterol across the mitochondrial membrane [44].

In summary, technical-grade glyphosate showed no acute or chronic effects on developing tadpoles, whereas tadpoles reared in environmentally relevant concentrations of Roundup Original and Roundup Transorb formulations suffered mortality and developmental abnormalities. Similar abnormalities were found on exposure to the surfactant POEA, and for all endpoints examined, POEA showed results similar to those with the Roundup Original formulation. Thus, our data indicate that the surfactant is not a biologically inert component of these pesticide formulations and that more research concerning the acute and chronic effects of surfactants as a group is required. Furthermore, a minimal concentration at which the animals were not affected by POEA, Roundup Original, and Roundup Transorb was not determined during the present study. In both 24- and 96-h exposures, calculated LC50 values indicated that Roundup formulations and POEA are more acutely toxic than the glyphosate technical material and newer formulations. These differences between formulations likely depend on the amount of POEA in the formulation, but surfactant information for some of these compounds can be difficult to obtain. Thus, more information is required on the type(s) of surfactants included with pesticide formulations and their fate in the environment. Finally, there should be consideration of the toxicity of components within herbicide formulations when making registration decisions on pesticides.

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