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ROUNDUP® AND AMPHIBIANS: THE IMPORTANCE OF CONCENTRATION, APPLICATION TIME, AND STRATIFICATION

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Abstract—The widespread use of pesticides raises the possibility that non-target organisms might also be affected. To assess this, the traditional approach has been to conduct short-term laboratory experiments spanning a range of lethal concentrations and some longer-duration experiments at sublethal concentrations. While this approach has been very useful, less attention has been paid to the timing of exposure and the impacts of multiple, small exposures versus single, large exposures. We examined the role of application amount, timing, and frequency using outdoor mesocosm communities containing larval amphibians (*Rana sylvatica* and *Bufo americanus*) and using a commercial formulation of the herbicide glyphosate (Roundup Original MAX®). Consistent with past studies, exposures of up to 3 mg acid equivalent (a.e.)/L caused substantial amphibian death. However, the amount of death was considerably higher when the herbicide was applied earlier in the experiment than later in the experiment. Single, large applications (at different times) had larger effects on tadpole mortality and growth than multiple, small applications (of the same total amount). The results may reflect an acclimation to the herbicide over time. In treatments with high tadpole mortality, there was no resulting increase in periphyton, suggesting that the reduction in tadpole herbivory might have been offset by direct negative impacts of the herbicide. We also discovered that temperature stratification caused herbicide stratification, with higher concentrations near the surface. Such stratification has important implications to the habitat choices of ectotherms that might prefer surface waters for thermoregulation or prefer deeper waters to avoid predators. Collectively, the present study demonstrates the importance of examining multiple applications times and frequencies to understand the impacts of pesticides on organisms. Environ. Toxicol. Chem. 2010;29:2016–2025. © 2010 SETAC

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INTRODUCTION

The application of pesticides to terrestrial and aquatic ecosystems is often a highly effective way of controlling pest species to improve agriculture, forestry, and human health. While the benefits of pesticides are well studied, less is generally known about the impact that individual pesticides might have on non-target organisms. For some of the most commonly applied pesticides, we have increasing knowledge regarding the impacts on non-target organisms based on traditional, short-term toxicity tests conducted in the laboratory and some longer-term studies conducted under natural or semi-natural mesocosm conditions [1,2].

A current challenge is to understand whether there are windows of time when organisms are particularly sensitive to pesticides, particularly in relation to when various pesticides are applied. By knowing the timing and magnitude of exposures along with the sensitivity of different life stages, we can achieve a much more refined assessment of risk. The importance of ontogenetic sensitivity has received little empirical attention, particularly in amphibians, yet the few studies that have been conducted (assaying only a few pesticides) often demonstrate that sensitivity can change over ontogeny, although there are currently too few studies to identify any predominant patterns in this sensitivity [3–6]. While it could be argued that the stages most sensitive to pesticides might be early in development or points in ontogeny that experience rapid development (i.e., metamorphosis), it remains unclear whether there are any general patterns among organisms or among pesticides. Hence, we need additional studies that examine how non-target organisms respond to particular concentrations of pesticides at different points in time.

When considering the timing of pesticide applications, a second challenge is to understand the impact of multiple, small applications to the environment versus single, large applications of the same total amount. Many pesticides are applied multiple times to the same site (either intentionally or via runoff, drift, and aerial transport) and the total concentration to which organisms are exposed depends upon the time between applications and the rate of pesticide breakdown. Given that multiple applications occur, we need to know how effects of multiple, small applications on non-target organisms compare to the effects of single, large applications that comprise the same total amount of added pesticide. To our knowledge, no studies in aquatic systems have directly addressed this question with an herbicide (see Relyea and Diecks [7] for a similar approach with the insecticide malathion).

We examined these questions using outdoor mesocosms containing larval amphibians that were exposed to a commercial formulation of glyphosate (Roundup Original MAX®) under different application rates and applications times. Amphibians are of particular toxicological and ecological importance given their high sensitivity to many pesticides and environmental changes [8] and because of recent correlations between the use of pesticides and the decline of amphibian populations [9]. In conducting this experiment, we tested the following hypotheses: increased concentrations of the herbicide will have increasingly negative effects on amphibian survival and growth;

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the herbicide will have similar effects on survival and growth when applied earlier in ontogeny than later in ontogeny; multiple, small applications of the herbicide will have the same effects as single, large applications of the same total amount.

MATERIALS AND METHODS

Pesticide background

The application of glyphosate-based herbicides has grown exponentially since its patent in 1975 and it is currently the most widely used group of herbicides in the world [10]. Glyphosate applications for U.S. agriculture have increased from 2.7 to 3.6 million kg in 1987 to 38.6 to 40.8 million kg in 2001 (http://www.epa.gov/oppbead1/pestsales/01pestsales/usage2001_2.htm). Its mode of action is to block the production of key amino acids used for protein synthesis and plant growth. Terrestrial formulations of glyphosate (e.g., Roundup®, Vision®; Monsanto, Saint Louis, MO, USA) also contain surfactants as part of their inert ingredients that improve the ability of glyphosate to penetrate the waxy cuticle on plant surfaces. Different formulations contain different surfactants, but a common and highly effective surfactant is polyethoxylated tallowamine (POEA), a derivative of animal fat. This is important because the POEA surfactant, while legally classified as an inert ingredient, is actually the component that causes high mortality to both fish and amphibians [11].

Although terrestrial formulations are not intended to be sprayed over water, several researchers have demonstrated that these formulations find their way into aquatic habitats either through drift or unintentional overspray [12]. Aquatic concentrations are measured in units of mg of acid equivalents per liter (mg a.e./L). Estimated worst-case-scenario glyphosate concentrations in freshwater range from 2.7 to 7.6 mg a.e./L [11,13,14] with observed values ranging up to 5.2 mg a.e./L in freshwater systems including streams, lakes, and wetlands [11,12,15]. In water, the half-life of glyphosate ranges from 7 to 8 d under laboratory conditions and 8 to 120 d in ponds, with temperature, pH, sorption and absorption all influencing its persistence [16]. The formulated product that we used, Roundup Original MAX, is a popular formulation for agricultural applications.

The mesocosm experiment

To examine the importance of glyphosate concentration and application time on larval amphibians, we conducted an outdoor mesocosm experiment at the Pymatuning Laboratory of Ecology in Pennsylvania. Mesocosm experiments represent semi-natural conditions and this experiment exposed two species of spring-breeding larval amphibians (from two families: wood frogs, *Rana sylvatica* [Ranidae] and American toads, *Bufo americanus* [Bufonidae]) to 12 different herbicide treatments in a completely randomized design. The first treatment was a no-pesticide control. The next nine treatments examined three concentrations of glyphosate (1, 2, and 3 mg a.e./L) that were applied once on either day 0, day 7, or day 14. The final two treatments applied glyphosate (0.33 or 1.00 mg a.e./L) repeatedly at three time points (on days 0, 7, and 14). In this case, we simulated events in which a wetland was contaminated on three occasions during a 3-week period, a scenario that could happen following accidental oversprays or drift combined with rain events that wash the herbicide off plant surfaces and into the water body [17,18]. Such a design can inform us of the effects of adding pesticides to communities at different times, but it cannot unambiguously determine whether the effects seen are due to differences in sensitivity over ontogeny or due to differences in the duration of exposure. Any tanks not receiving glyphosate on days 0, 7, or 14 were mock-dosed to equalize disturbance. The 12 treatments were replicated four times for a total of 48 mesocosms.

The mesocosms were 750-L cattle tanks, filled with approximately 542 L of well water on April 28-29, 2007. To each mesocosm, we added 15 g of rabbit chow as an initial nutrient source, 200 g of oak (*Quercus* spp.) leaf litter to serve as a natural substrate for periphyton growth, and two aliquots of local pond water containing zooplankton, phytoplankton, and periphyton. In this way, we constructed semi-natural food webs that share several components of natural ponds and wetlands. Two ceramic tiles (15×15 cm) were placed into each tank to assess periphyton growth. Mesocoms were allowed 2 weeks to develop algal and zooplankton communities before the amphibians were introduced. All mesocosms were covered with 60% shade cloth lids to prevent other organisms from ovipositing into the water.

The wood frogs and American toads were collected as egg masses from nearby ponds (20 clutches of wood frogs and 10 clutches of American toads) and hatched in covered culture pools filled with aged well water. The hatchlings were fed rabbit chow ad libitum until used in the experiment. Once both tadpole species became large enough to safely handle, we mixed the hatchlings of all clutches and selected tadpoles for the experiment from this mixture. Both species of tadpoles were early in development. The initial developmental stage [19] and mean mass ($\pm$ 1 standard error) was stage 26 and 106 ± 7 mg for wood frogs and stage 25 and 17 ± 2 for American toads. Every tank received 20 wood frog tadpoles and 20 American toad tadpoles. This density, 8 individuals/m^2, is well within natural levels (R. A. Relyea, personal observation).

Following the addition of both tadpole species on May 18, 2007 (defined as day 0), we applied the herbicide treatments. To obtain nominal concentrations of 0, 0.33, 1, 2, and 3 mg a.e./L of glyphosate, we added 0, 0.333, 1, 2, and 3 mL of Roundup Original MAX which contains 48.7% glyphosate active ingredient (a.i.) plus inert ingredients that include a surfactant. Although the surfactant is reportedly not POEA (S. Mortensen, Monsanto, personal communication), past research has determined that the toxicity of this formulation to larval amphibians is nearly identical to other glyphosate formulations that do contain POEA [20,21]. The herbicide was dissolved into a half-liter container of water that was then drizzled across the surface of the water. The surface water was then mixed to help distribute the herbicide.

Approximately 3 h after dosing, water samples were taken to determine the actual concentration of glyphosate in the water and to determine whether the concentrations of the herbicide differed between the top and bottom of the water column. We took water samples from the four replicates of each treatment from both the top and bottom of the water column using a tube sampler. Water samples from the four replicate tanks were pooled and placed into a pre-cleaned, glass amber jar and held at 2°C to prevent breakdown. Water from the control (0 mg a.e./L) treatments was not tested, but past well water tests have found no traces of glyphosate. Water samples were sent to Mississippi State Chemical Laboratory for chemical analysis. The Laboratory filtered the samples, so the concentrations reported represent the amount of glyphosate dissolved in the water.

The tested water samples indicated that the herbicide concentrations differed between the top and bottom of the water column (Table 1). For all treatments receiving single applica-

Table 1. Nominal versus observed concentrations of glyphosate from the top and bottom of outdoor pond mesocosms. All concentrations are reported as milligram acid equivalent per liter (mg a.e./L). Glyphosate was added using the commercial formulation Roundup Original MAX® (Monsanto, USA). For two of the treatments (0.33 and 1.00 mg a.e./L), a second application occurred on day 7 and a third application occurred on day 14.

Application Date	Nominal concentration	Observed top concentration	Observed bottom concentration	Observed mean concentration
Day 0	0.33	0.42	0.15	0.28
	1.00	1.46	0.37	0.91
	2.00	3.10	0.81	1.96
	3.00	4.77	1.33	3.05
Day 7	0.33×2	0.71	0.39	0.55
	1.00×2	2.42	1.40	1.91
Day 14	0.33×3	0.83	0.56	0.70
	1.00×3	3.09	2.11	2.60

tions on day 0, the mean observed concentrations from the top and bottom water samples were very close to the target concentrations and, for simplicity, will be referred to as 0, 0.33, 1, 2, and 3 mg a.e./L. Later single applications were assumed to have the same concentrations as early single applications of the same amount and thus were not tested. For treatments receiving multiple applications of glyphosate, a second and third set of samples were tested to determine if the second and third applications caused an accumulation of glyphosate (Table 1). These samples indicated that the three applications accumulated to a concentration that was 70 to 87% of the nominal concentration, thus demonstrating relatively little herbicide breakdown. The mass and developmental stages of the tadpoles at the midway and late application times were not quantified because capturing and handling these animals, and then returning them to the mesocosms, posed the potential for adding an additional stress to the animals that could potentially interact with the treatments that we applied.

Water quality and periphyton

Water quality measurements were taken to assess the abiotic conditions of the experiment. Temperature and pH were measured at the top and bottom of the mesocosm (5 cm below the water's surface and 5 cm above the bottom of the tank, respectively). Dissolved oxygen was taken midway in the water column. Water quality measurements were taken twice during the experiment (before glyphosate application on day 7 and day 14) using a multimeter probe that was calibrated before each use (Multiline P4 Universal Meter, WTW).

To obtain insight into periphyton abundance over time, the periphyton was assessed on day 12 and day 17. Ceramic tiles were removed from mesocosm tanks, scrubbed for periphyton, and rinsed with filtered well water. The resulting slurry was vacuum-filtered through a predried (80°C, 24 h) and preweighed Whatman® GF/C filter. The filters were dried (80°C, 24 h) and weighed again after samples were filtered to quantify periphyton biomass. The difference in initial and sample weights of the filter provided an estimate of periphyton abundance within each mesocosm.

The experiment was terminated on day 18, because the toads were about to initiate metamorphosis and lose considerable mass. On this day, we removed the water and leaf litter from each tank and collected all surviving amphibians. Amphibians were euthanized with MS-222 and then preserved in 10% formalin. Preserved animals were later counted and weighed to determine the survival and growth of the two species. The survival of each species from each tank and the mean mass of individuals from each mesocosm served as the amphibian response variables. The average developmental stage of the tadpoles [19] at the end of the experiment was 38.2 for toads and 37.8 for wood frogs. We did not analyze the developmental stages among treatments because changes in tadpole development simply tracked changes in tadpole growth.

Statistical analysis

We used analysis of variance to determine the effects of application amount and timing on the survival and mass of wood frogs and American toads. We began by conducting a multivariate analysis of variance (MANOVA) across all 12 treatments to determine if there was a significant multivariate effect of the treatments. Following a significant multivariate effect, we conducted separate univariate analyses of variance (ANOVAs) on the four response variables (wood frog survival and mass, toad survival and mass). Analyses of mass did not include a covariate of survival because there was no indication that tanks with reduced densities experienced greater growth. For significant univariate effects, we tested several hypotheses using planned mean comparisons. To determine the lowest-observable-effect concentration (LOEC), we used a Dunnett test that compared the control treatment to each treatment containing glyphosate. To determine whether multiple small applications differed from single, larger applications at three different times, we conducted Tukey mean comparison tests. We also estimated the concentrations that would cause 10, 50 and 90% mortality (LC10, LC50, and LC90, respectively) for each species at each application time using standard probit analyses. This included estimates of the 84% confidence intervals for each probit analysis. Simulations have shown that non-overlapping 84% confidence intervals are different at $\alpha = 0.05$ [22].

We also assessed the assumptions of the analyses. The survival and mass data were approximately normal without having to be transformed. A few of the treatments had low variance (e.g., control wood survival 100% toad survival 98%) or high variance (e.g., toad mass early application 3 mg a.e./L), but ANOVAs tend to be robust to violations of this assumption [23]. For toad mass, the variance for the early 3 mg a.e./L was substantially greater than all other treatments, likely due to higher death and, hence, fewer individuals and replicates. The analysis was run both with and without this treatment and the interpretation of the results was not altered. Thus, we left the treatment in the analysis. Rank transformation is one established method of analyzing these data, however we chose not to rank-transform the data because doing so dramatically altered the relative survival values in a way that obfuscated obvious interactive treatment effects.

To test if concentration and application time had interactive effects on tadpole survival and mass, we analyzed a subset of

Table 2. Univariate (analysis of variance) results using Dunnett's test (control versus treatments containing glyphosate) and Tukey's test (multiple versus single applications). Treatments are labeled using glyphosate concentration (mg a.e./L) and time of application.

	Wood survival	Toad survival	Wood mass	Toad mass
Treatment	<0.0001	<0.0001	0.0010	<0.0001
Control vs.:				
1 Early	1.0000	0.7968	1.0000	0.1239
2 Early	<0.0001	0.0253	0.6358	0.5465
3 Early	<0.0001	<0.0001	0.9933	0.7582
1 Midway	0.9027	1.0000	0.2472	0.9130
2 Midway	0.0415	0.2770	0.1080	0.0067
3 Midway	<0.0001	<0.0001	0.9936	0.0003
1 Late	1.0000	1.0000	0.6863	0.9999
2 Late	0.3699	0.9851	0.0004	0.0006
3 Late	<0.0001	0.0074	<0.0001	<0.0001
0.33 X 3	0.9828	1.0000	0.8310	0.7495
1 X 3	0.5479	0.9998	0.5079	0.9924
0.33 × 1 applications versus:				
1 Early	0.1296	0.6110	0.7894	0.3715
1 Midway	0.7735	1.0000	0.7688	0.9742
1 Late	0.1296	0.9986	0.9956	0.5424
1 × 3 applications versus:				
3 Early	<0.0001	<0.0001	0.8743	0.9975
3 Midway	<0.0001	<0.0001	0.9999	0.0959
3 Late	0.0080	0.0879	0.0251	0.0100

the data that only contained the three concentrations (1, 2, or 3 mg a.e./L) that were applied at three different times (day 0, 7, or 14). We began with a MANOVA followed by four ANOVAs to test the main effects and concentration-by-application time interactions. The survival and mass data for this subset of treatments generally met the assumptions of normality and equality of variance. Significant ANOVAs were followed by mean comparisons using a Tukey mean comparison test.

Measurements of periphyton and water quality were separately analyzed with repeated-measures ANOVAs (rm-ANOVAs) since they were measured on multiple days. For variables that were measured at two water depths (pH and temperature at the top and bottom of the mesocosm), the data were analyzed by rm-ANOVAs while nesting sample depth within each treatment. When treatment effects were detected, planned contrasts were conducted to compare all treatments to the control. In conducting the planned contrasts, we took advantage of the fact that any treatments that had not yet received an application of Roundup at the time of the measurements remained identical to the control mesocosms. For example, when we measured pH and temperature on day 7, just prior to applying the midway Roundup treatment, all mesocosms assigned the midway and late applications remained identical to the control treatment. Therefore, planned contrasts could pool the midway- and late-application treatments with the control treatment. The data were log-transformed to achieve normality and homogeneity of variances.

RESULTS

Amphibian survival and growth

There was a significant multivariate effect of the 12 treatments on amphibian survival and mass (Wilks' $F_{44,117} = 8.1$, $p < 0.0001$). Survival in the control treatment was high for both wood frogs (100%) and toads (98%; Table 2, Fig. 1). For wood frogs, survival was reduced with early applications of 2 or 3 mg a.e./L, midway applications of 2 or 3 mg a.e./L, and late applications of 3 mg a.e./L. Similarly, for American toads, survival was reduced with early applications of 2 or 3 mg a.e./L, midway applications of 3 mg a.e./L, and late applications of 3 mg a.e./L. Thus, Roundup's impacts on tadpole survival varied with application time and concentration. Next, we quantified the LC10, LC50, and LC90 values (Table 3) to determine whether the values differed (based on non-overlapping LC50 84% confidence intervals). For both species of tadpoles, the LC50 estimates for early and midway applications were similar to each other, but significantly lower than the LC50 estimates for late applications.

Individual tadpole mass exhibited fewer treatment differences than tadpole survival (Table 2; Fig. 2). For wood frogs, mass was only reduced when exposed to late applications of 2

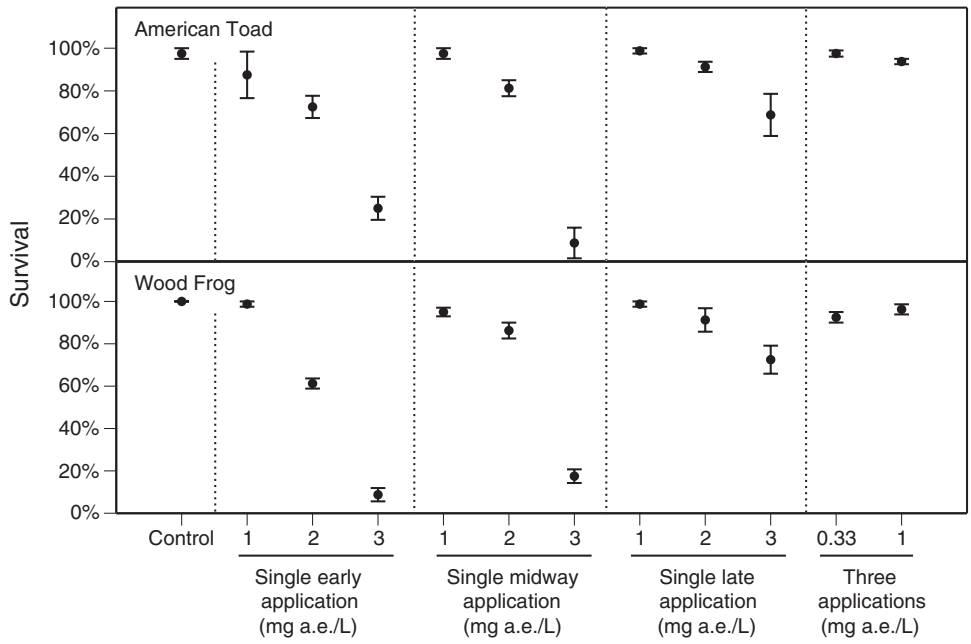

Fig. 1. Survival of American toad and wood frog tadpoles when exposed to varying Roundup Original MAX® concentrations (mg a.e. of glyphposate/L) at different times (day 0, 7, or 14). Data points represent mean survival (± 1 standard error) for all four replicates. Survival was recorded on day 18 following experimental takedown.

Table 3. Results of probit analyses used to estimate the LC10, LC50, and LC90 values (lethal concentrations that cause 10, 50, and 90% mortality) for Roundup Original Max® in outdoor mesocosms at three application times. Means are followed by 84% confidence intervals; non-overlapping confidence intervals are significantly different ($\alpha = 0.05$) [22].

Species	Application	LC10	LC50	LC90
Wood frog	Early (day 0)	1.45 (1.29, 1.57)	2.10 (2.00, 2.19)	3.03 (2.83, 3.34)
	Midway (day 7)	1.56 (1.07, 1.84)	2.44 (2.15, 2.79)	3.80 (3.20, 5.70)
	Late (day 14)	2.02 (1.47, 2.34)	4.27 (3.47, 7.42)	9.03 (5.83, 32.9)
American toad	Early (day 0)	0.99 (0.42, 1.35)	2.31 (1.86, 3.06)	5.36 (3.74, 15.2)
	Midway (day 7)	1.67 (0.72, 2.01)	2.30 (1.84, 2.89)	3.18 (2.63, 7.39)
	Late (day 14)	1.98 (1.49, 2.28)	3.93 (3.33, 5.83)	7.81 (5.44, 20.3)

or 3 mg a.e./L. For American toads, mass was reduced when exposed to midway or late applications of 2 or 3 mg a.e./L. Thus, the herbicide's effect on tadpole growth varied with application time and concentration, tending to have larger effects later in the experiment.

We also compared the effects of three smaller applications to one larger application that was added early, midway, or late in the experiment. In comparing a single application of 1 mg a.e./L to three applications of 0.33 mg a.e./L, there were no differences in survival or mass of the two anuran species (Table 2; Figs. 1–2). However, in comparing a single application of 3 mg a.e./L to three applications of 1 mg a.e./L, the single application caused lower survival of both species, regardless of when the single larger application was applied (although the late application of 3 mg a.e./L was not significant; $p = 0.088$). In terms of individual mass, both species were smaller when exposed to the single, larger application, but only when it was applied late in the experiment.

In the subset of nine treatments (three concentrations at three application times), the MANOVA found significant effects of glyphosate concentration, application time, and their interaction (Table 4, Figs. 1–2). Subsequent univariate analyses indicated that the main effects and their interactions were significant (or marginally non-significant; $p = 0.055$) for all four response variables (Table 5). Mean comparisons indicated that, for wood frogs, increasing the concentration from 1 to 3 mg a.e./L caused a 91% decline in survival when applied early ($p < 0.0001$) and a 82% decline in survival when applied midway ($p < 0.0001$), but only a 27% decline in survival when applied late in the experiment ($p = 0.0008$). For American toads, increasing the concentration from 1 to 3 mg a.e./L caused a 75% decline in survival when applied early ($p < 0.0001$) and an 91% decline in survival when applied midway ($p < 0.0001$), but only a 31% decline in survival when applied late in the experiment ($p = 0.059$).

Individual tadpole mass also showed interactive effects of concentration and application time that were either significant or marginally non-significant (Table 5; Fig. 2). For wood frogs, mean comparisons indicated that increasing the concentration of glyphosate from 1 to 3 mg a.e./L caused no effect early in ontogeny ($p = 1.00$) or midway in ontogeny ($p = 0.99$), but a

Table 4. Results of a multivariate analysis of variance examining the effects of glyphosate concentration and time of application on tadpole mass and survival.

Multivariate test	*df*	*F* value	*p* value
Application timing	8,42	9.3	<0.0001
Glyphosate concentration	8,42	21.1	<0.0001
Timing · Concentration	16,65	4.7	<0.0001

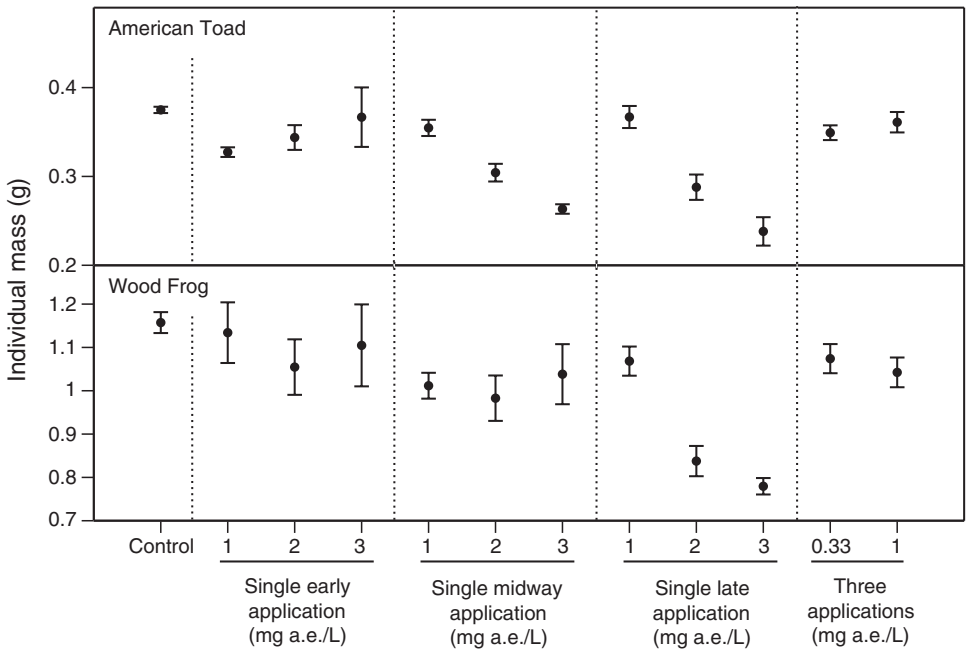

Fig. 2. Mean individual mass of American toad and wood frog tadpoles exposed to varying Roundup Original MAX® concentrations (mg a.e. of glyphposate/L) at different times (day 0, 7, or 14). Data points represent mean individual mass (± 1 standard error) among the four replicates. Individual mass was recorded on day 18 following experimental takedown.

Table 5. Univariate test results (p values) of significant main effects of time of application, glyphosate concentration, and the interaction.

Univariate tests	Application timing	Glyphosate concentration	Timing · concentration
Wood Survival	<0.0001	<0.0001	<0.0001
Toad Survival	0.0002	<0.0001	0.019
Wood Mass	0.0004	0.053	0.055
Toad Mass	0.008	0.0002	0.0013

27% decline in mass late in ontogeny ($p < 0.024$). For American toads, increasing the concentration of glyphosate from 1 to 3 mg a.e./L caused no change in mass early in the ontogeny ($p = 0.99$), but cased a 16% decline in mass midway in ontogeny ($p = 0.037$) and a 35% decline in mass late in ontogeny ($p < 0.0001$).

Periphyton and water quality

The analysis of periphyton mass exhibited an effect of time ($F_{1,36} = 21.4$, $p < 0.001$), but no treatment ($F_{11,36} = 1.5$, $p = 0.19$) or time-by-treatment interaction ($F_{11,36} = 1.1$, $p = 0.37$). The biomass of periphyton increased over time across all treatments.

The analysis of dissolved oxygen exhibited an effect of treatment ($F_{11,36} = 4.6$, $p < 0.001$), time ($F_{1,36} = 9.8$, $p = 0.004$), and their interaction ($F_{11,36} = 2.6$, $p = 0.016$; Fig. 3). On both day 7 and day 14, separate ANOVAs detected significant treatment effects ($F_{11,36} = 2.1$, $p = 0.048$; $F_{11,36} = 4.4$, $p = 0.003$, respectively). On day 7, none of the treatments differed from the control (all $p > 0.05$), but the early application of 3 mg a.e./L caused 23% higher oxygen than the early application of 1 mg a.e./L ($p < 0.002$). On day 14, midway applications of 2 and 3 mg a.e./L had higher oxygen concentrations than the control ($p < 0.001$). In addition, early applications of 3 mg a.e./L continued to have higher oxygen concentrations than the early application of 1 mg a.e./L ($p = 0.003$).

The analysis of temperature exhibited an effect of treatment ($F_{11,36} = 2.3$, $p = 0.03$), depth ($F_{1,36} = 3547$, $p < 0.0001$) and time ($F_{1,36} = 3745$, $p < 0.0001$; Fig. 4). Among the possible interactions, there was no depth-by-treatment interaction ($F_{11,36} = 1.7$, $p = 0.12$), but there were time-by-treatment ($F_{11,36} = 3.1$, $p = 0.005$), time-by-depth ($F_{1,36} = 175$, $p < 0.0001$), and time-by-treatment-by-depth ($F_{11,36} = 3.5$, $p < 0.002$) interactions. As a result, we analyzed the data within each sample date. On day 7, there was a depth effect ($F_{1,36} = 2263$, $p < 0.0001$), but no treatment effect ($F_{11,36} = 1.0$, $p = 0.47$) or treatment-by-depth interaction ($F_{11,36} = 0.5$, $p = 0.87$). At this time, the water near the bottom of the mesocosm was approximately 3°C colder than the water near the top of the mesocosm. On day 14, there was a depth effect ($F_{1,36} = 3138$, $p < 0.0001$), treatment effect ($F_{11,36} = 6.2$, $p = 0.0001$), and a depth-by-treatment interaction ($F_{11,36} = 3.4$, $p = 0.002$). The interaction occurred because temperatures near the bottom of the mesocosm exhibited weaker treatment effects ($F_{11,36} = 2.3$, $p = 0.027$) than temperatures near the top ($F_{11,36} = 7.5$, $p < 0.0001$). Temperature differences among treatments at a particular depth were typically less than 1°C whereas the difference between top and bottom temperatures was approximately 4°C. Collectively, the data indicate that the mesocosms were colder near the bottom than the top.

The analysis of pH showed no effect of treatment ($F_{11,36} = 1.5$, $p = 0.19$), but an effect of depth ($F_{1,36} = 130$, $p < 0.0001$) and time ($F_{1,36} = 19$, $p < 0.0001$; Fig. 4). There also was a time-by-treatment interaction ($F_{11,36} = 2.4$, $p = 0.02$), but no treatment-by-depth ($F_{11,36} = 1.3$, $p = 0.27$), time-by-depth ($F_{1,36} = 0.2$, $p = 0.65$), or time-by-depth-by-treatment ($F_{11,36} = 1.5$, $p = 0.17$) interactions. Because of these interactions, we examined the data within each sample time. On day 7, pH showed a marginal effect of treatment ($F_{11,36} = 2.0$, $p = 0.055$), an effect of depth ($F_{1,36} = 137$, $p < 0.0001$), and a depth-by-treatment interaction ($F_{11,36} = 2.8$, $p = 0.009$). On day 14, there was an effect of depth ($F_{1,36} = 70$, $p < 0.0001$), but no effect of treatment ($F_{11,36} = 1.8$, $p = 0.1$) or a depth-by-treatment interaction ($F_{11,36} = 0.7$, $p = 0.76$). On both day 7 and 14, pH was slightly higher near the top of the mesocosm (a difference of 0.08 pH units). Thus, differences in pH between the top and bottom of the mesocosm were small.

DISCUSSION

The results of the present study indicate that Roundup Original MAX, a herbicide containing glyphosate and an

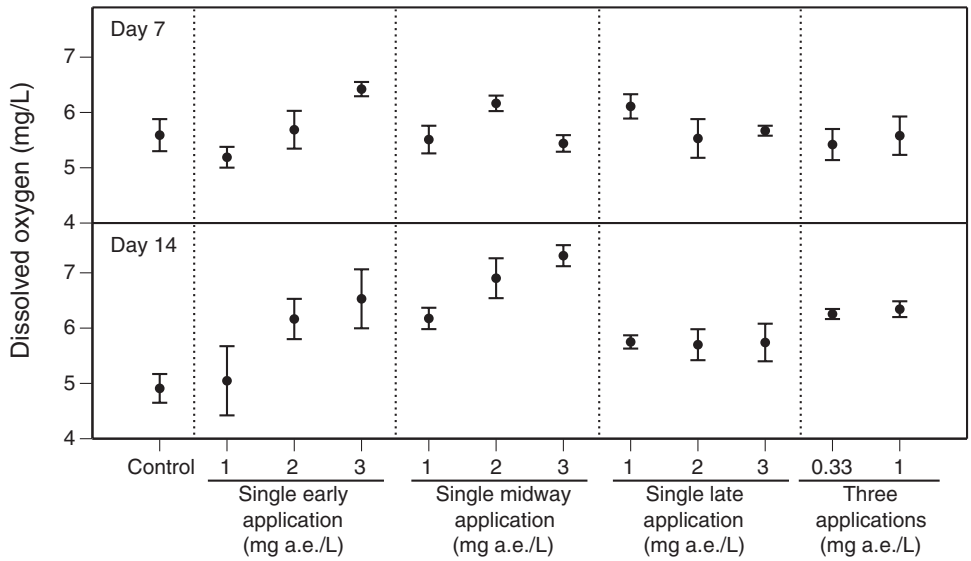

Fig. 3. Concentrations of dissolved oxygen measured on days 7 and 14 in mesocosms exposed to varying Roundup Original MAX® concentrations (mg a.e. of glyphposate/L) at different times (day 0, 7, or 14). Data points represent means (± 1 standard error).

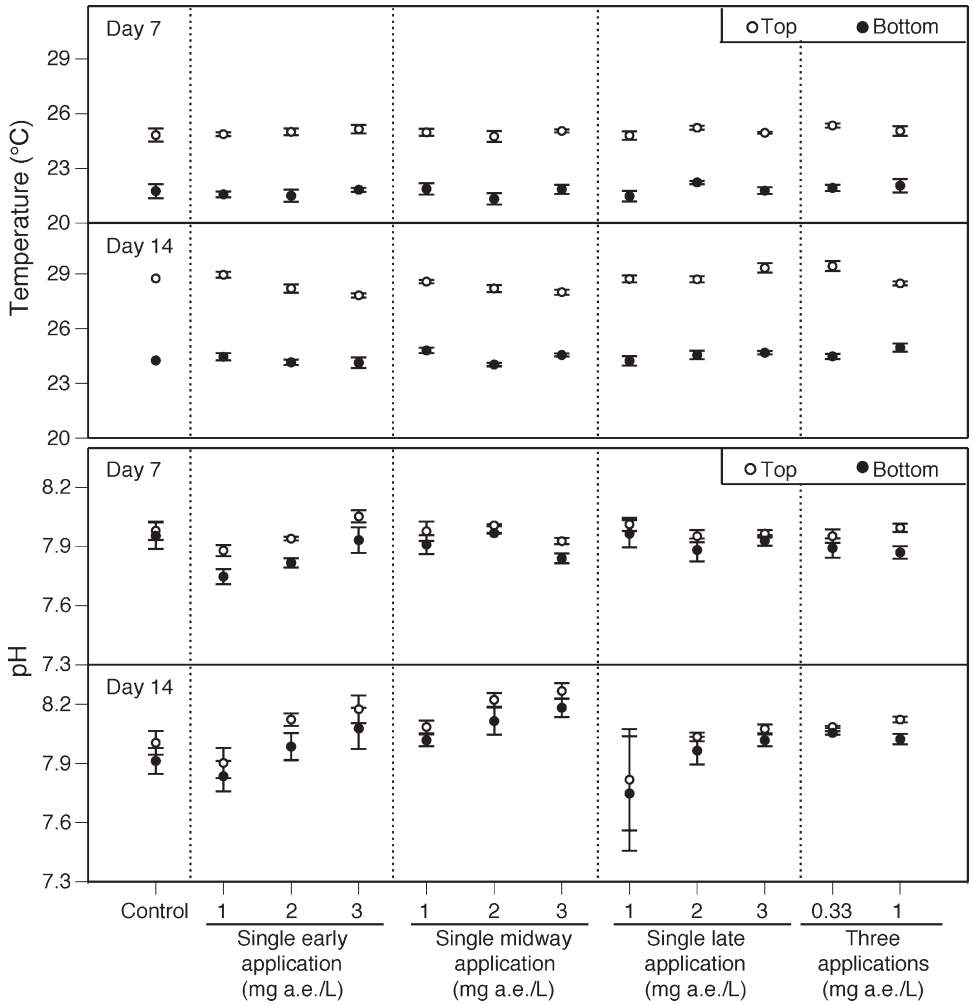

Fig. 4. The temperature and pH of outdoor mesocosms measured on days 7 and 14 at the top and bottom of the water column. Data points represent replicate means (± 1 standard error) of mesocosm tanks dosed with varying Roundup Original MAX® concentrations (mg a.e. of glyphposate/L) at different times (day 0, 7, or 14).

undisclosed surfactant, can impact wetland communities and these effects depend on application amount, timing, and frequency. By the end of the experiment, multiple smaller applications caused weaker effects than single applications of the same total concentration, even though the multiple, smaller concentrations showed little evidence of breakdown over time. In addition, the temperature in the water column stratified and this lead to the stratification of the herbicide in the water column.

The lethality of Roundup occurred under concentrations that have been observed in nature and the results are consistent with several past studies. For example, recent laboratory studies have been conducted on nine species of tadpoles from North America using Roundup Original MAX [21]. The $LC50_{4\text{-d}}$ values (the concentration that would kill 50% of a population) ranged from 0.8 to 2.0 mg a.e./L and the $LC90_{4\text{-d}}$ values (the concentration that would kill 90% of a population) ranged from 1.2 to 2.8 mg a.e./L. Similar values have been found in eight species of tadpoles from Colombia using Glyphos + Cosmo-Flux (i.e. glyphosate and the POEA surfactant) [24]. Given that these 17 species were all tested early in ontogeny, it is not surprising in the present study that a single application of 3 mg a.e./L early in ontogeny caused 75% of American toads and 91% of wood frogs to die. As reviewed in Relyea and Jones [21], the high lethality of Roundup Original MAX, which has an undisclosed surfactant, is very similar to a series of studies that have used formulations of Roundup containing the popular POEA surfactant. Particularly under higher pH conditions (for reasons that are unclear), glyphosate formulations containing POEA have caused substantial amphibian mortality under laboratory, mesocosm, and pond enclosure experiments [20,25–31].

While previous studies led to the expectation of high rates of death at the highest concentration of glyphosate used in the present study, a striking observation was that this impact declined between early and late applications of the herbicide. Single early applications killed 91% of wood frogs and 75% of toads whereas single late applications killed 27% and 31% of the animals, respectively. There are two potential mechanisms to explain this observation: it could be that tadpoles are more sensitive to the herbicide early in ontogeny than late in ontogeny; or tadpoles receiving the single early application were exposed to the herbicide for a longer period of time (18 d) than tadpoles receiving the single late application (4 d). The design employed cannot distinguish between these two alternatives. However, in previous work employing laboratory and mesocosm experiments (using tadpoles spanning a range of sizes), we have observed that the vast majority of death from exposure to commercial formulations of the herbicide occur within the first 1 to 3 d [20,21], although we do not have experimental data lasting the same duration as the current experiment. Of course, it

may also be that both mechanisms are operating simultaneously and future experiments should examine each process in isolation. What is clear is that if a wetland community containing wood frogs and American toads were to become contaminated by the herbicide, contamination early in the spring would have a higher lethal effect on the tadpoles than contamination later in the spring.

Relatively little is known about the sensitivity of larval amphibians to pesticides over ontogeny. In what appears to be the only direct investigation with glyphosate, Howe et al. [27] found green frog (*R. clamitans*) tadpoles very early in ontogeny (Gosner stage 20) were more tolerant of glyphosate formulations than tadpoles that were a bit more developed (Gosner stage 25; similar to the initial developmental stage of the tadpoles in the present experiment). Later developmental stages, as would have been present at the time of the late application, were not examined in that study. Similar patterns of lower sensitivity very early in development (Gosner stage 3) compared to later in development (Gosner stages 19 and 25) have been found in the effects of copper sulfate on *Bufo calamita* tadpoles [6]. Studies conducted later in ontogeny, as in the present study, have found mixed results including increased sensitivity later in ontogeny [5], or similar sensitivity over ontogeny [32]. Two other studies on amphibians have conducted exposures at different times during ontogeny (without controlling exposure duration, similar to the present study) and found decreased sensitivity to pesticides when applications were conducted later in ontogeny [3,4]. Because these few studies span different species and pesticides that have very different modes of action, much more work needs to be done before we arrive at any strong generalities regarding the spectrum of lethal effects of pesticides on amphibian larvae exhibited throughout their ontogeny. In those systems where ontogenetic changes in susceptibility have been found, it might be insightful to determine the mechanisms underlying the changes in sensitivity during development.

The inclusion of treatments with multiple applications over time allowed us to investigate whether amphibian larvae were more affected by single large applications or several small applications over time that sum to the same total amount of pesticide. In the lowest concentration comparison, neither a single application of 1 mg a.e./L or three applications of 0.33 mg a.e./L caused any amphibian death. Thus, both application scenarios were sublethal. In contrast, single applications of 3 mg a.e./L always caused significant mortality (at all application times), whereas three applications of 1 mg a.e./L over time caused no significant mortality. However, one cannot conclude that because the tadpoles did not die that they were unaffected by chronic exposure to glyphosate formulations. Howe et al. [27] reported leopard frogs (*R. pipiens*) that were exposed to 0.6 and 1.8 mg a.e./L of Vision® (glyphosate plus the POEA surfactant) for 42 d experienced an increased time to metamorphosis, reduced size at metamorphosis, altered gonads, tail damage, and increased thyroid hormone production.

There are several possible explanations for the observation that three applications of 1 mg a.e./L caused no mortality while single applications of 3 mg a.e./L, added at different time points in the experiment, caused significant mortality. First, it could be that when the herbicide was added as three applications over time, the herbicide degraded sufficiently between applications such that the final concentration was below the lethal range. However, the water tests confirmed that this was not the case; the mean concentration of glyphosate in tanks receiving three applications of 1 mg a.e./L was 0.9 mg a.e./L after the first application, 1.9 mg a.e./L after the second application, and 2.6 mg a.e./L after the third application. Thus, the final concentration from three applications over time was 87% of the single late application (3 mg a.e./L), suggesting minimal herbicide degradation. This slow breakdown of glyphosate is consistent with that observed in the multiple applications of 0.33 mg a.e./L over time as well as in previous studies [33].

When comparing different exposure regimes over time, it is recommended that one considers both peak concentrations and the area under the concentration versus time curve (it is not recommended that one considers the time weighted average if one suspects that there are differentially sensitive stages during the experiment; http://elink-info.unicatt.it/ELINK_Executive_Summary.pdf [34]). Using this approach (and the evidence of minimal herbicide breakdown), we can compare the three applications of 1 mg a.e./L versus a single early application of 3 mg a.e./L. The single early application of 3 mg a.e./L had a peak concentration of 3 mg a.e./L and an area under the curve of 54 mg a.e. · d/L (calculated as 3 mg a.e./L · 18 d). In contrast, the three applications of 1 mg a.e./L had a peak concentration (late in the experiment) of 2.6 a.e./L and an area under the curve of 30 mg a.e. · d/L (calculated as [0.9 mg a.e./L · 7 d] + [1.9 mg a.e./L · 7 d] + [2.6 mg a.e./L · 4 d]). Thus, the higher mortality observed with the single early application could be due to the 15% higher peak concentration or due to the 80% greater overall exposure.

Next we can compare the three applications of 1 mg a.e./L versus a single midway application of 3 mg a.e./L. In this case, the single midway application of 3 mg a.e./L would have a peak concentration of 3 mg a.e./L and an area under the curve of 33 mg a.e. · d/L (calculated as 3 mg a.e./L · 11 d). Given that the three applications of 1 mg a.e./L had a peak concentration (late in the experiment) of 2.6 a.e./L and an area under the curve of 30 mg a.e. · d/L, the higher mortality caused by the single midway concentration could be due to the 15% higher peak concentration or due to the 10% greater area under the curve.

Finally, we can compare the three applications of 1 mg a.e./L versus a single late application of 3 mg a.e./L. In this case, the single late application of 3 mg a.e./L would have a peak concentration of 3 mg a.e./L and an area under the curve of 12 mg a.e. · d/L (calculated as 3 mg a.e./L · 4 d). Thus, the higher mortality caused by the single late application could be due to the 15% higher peak concentration but not due to the 60% smaller area under the curve.

In summary, the above three comparisons suggest that the higher mortality observed with the single versus multiple concentrations is consistent with the mechanism of higher peak concentration but not consistent with the mechanism of higher total exposure. As noted, however, the difference in peak concentrations in all cases was rather small (15%). Moreover, in the case of the single late application, one would expect amphibians exposed to three applications of 1 mg a.e./L to experience mortality levels that were intermediate to late applications of 2 and 3 mg a.e./L. However, this was not observed. One intriguing possibility is that exposure to 1 mg a.e./L early in the experiment and an additional 1 mg a.e./L midway in the experiment may have acclimated the tadpoles to the herbicide's effects. Of course, further empirical tests are needed to validate this hypothesis. Unfortunately, there appear to be no other studies that directly compare multiple small exposures to an equivalent single large exposure of pesticides in either amphibians or fish. However, a similar type of study by Relyea and Diecks [7] found that 7 weekly applications of the insecticide malathion (at 10 ppb) had larger effects than single larger

applications (50 or 250 ppb). In this case, the mechanism was via an indirect effect through the foodweb; weekly applications prevented the zooplankton assemblage from recovering, thereby promoting a phytoplankton bloom that reduced periphyton abundance and slowed the growth and development of tadpoles.

Exposure to the herbicide not only affected tadpole survival, but also affected tadpole growth. Reduced tadpole mass was observed under the highest two concentrations of glyphosate and only when glyphosate was applied midway (toads) or late in the experiment (toads and wood frogs). This is particularly interesting, since the low survival in 2 and 3 mg a.e./L treatments (i.e., as low as 9%), should have provided the surviving individuals greater per-capita food (i.e., periphyton), thus allowing the survivors to grow larger. We found no significant impacts of the pesticide treatments on periphyton, suggesting that the positive effects of reduced herbivory (due to tadpole mortality) may have been counter-balanced by direct negative impacts of glyphosate on algal growth, thereby producing no net change in algal biomass relative to the control. Thus, the herbicide not only impacted the tadpoles, but also appears to have affected the periphyton. Previous studies have confirmed the ability of glyphosate to negatively affect algal growth [35,36].

The negative effects on tadpole growth observed under the higher herbicide concentrations are supported by past studies. An experiment using 1 mg a.e./L of glyphosate also found no effects on the growth of leopard frog (*R. pipiens*), gray tree frog (*H. versicolor*), and American toad tadpoles [31]. Similarly, in 16-d laboratory studies, tadpoles of several species (including toads) experienced no reduction in growth relative to a control when fed a constant ration of fish flakes and exposed to 0.75 mg a.e./L. In the same experiment, however, there were significant reductions in growth when exposed to 1.5 mg a.e./L [37]. This suggests that even when food is equally available among treatments with different concentrations of herbicide, tadpoles exposed to 1.5 mg a.e./L still grow more slowly. Therefore, it appears that the reduced growth observed in the current experiment occurred not because the tadpoles had less food available, but because the herbicide directly altered tadpole behavior or physiology in ways that caused slower growth. Slower growth can result in metamorphic frogs that are either smaller at the time of metamorphosis or take longer to metamorphose. Such outcomes are associated with reduced survival to sexual maturity, delayed time to maturity, reduced size at maturity, decreased mating success, lower female fecundity, and smaller eggs [38,39].

Quantifying the water quality variables (pH, temperature, and dissolved oxygen) not only documented the abiotic conditions of the experiment, but also documented that the water column was stratified, particularly for temperature. This is a common limnological observation and thermal stratification can make the water column resistant to the mixing of many other compounds [40]. This stratification, in turn, appears to be responsible for the observation that the tested concentrations of glyphosate did not mix evenly throughout the water column. Instead, concentrations were considerably greater near the surface than near the bottom of the mesocosms. In short, the herbicide became stratified because water temperature was stratified, an observation that has been observed in at least two other studies [41,42]. As Clark et al. [43] noted in their review of coastal wetlands, the occurrence of pesticide stratification has important implications to the exposure that organisms actually experience. For example, ectotherms (including larval amphibians) typically seek the warmer temperatures of surface waters [44]. In doing so, they will inadvertently be exposed to higher than average concentrations. This may be the reason that researchers working in much shallower mesocosms (water depth = 15 cm) find lower rates of mortality [45]. Shallow pools would likely have more evenly distributed concentrations of the herbicide whereas deeper pools that could stratify would produce higher than nominal concentrations near the surface, thereby causing higher rates of death if the animals swam near the surface. However, if other risks such as predators also prefer to live in the surface waters, this may scare prey organisms into deeper waters that have less lethal concentrations of pesticides. The presence of pesticide stratification means that testing water samples from higher up in the water column will produce higher than nominal concentrations whereas testing water samples from lower in the water column can produce much lower concentrations than nominal concentrations.

CONCLUSION

The present study demonstrated that the herbicide Roundup Original MAX, composed of glyphosate and a surfactant, caused a range of mortality and growth effects that were dependent upon both the concentration and the timing of the application. We clearly need many more studies of pesticide applications over ontogeny to determine whether these effects are due to differences in exposure duration or differences in sensitivity over ontogeny. We also observed that multiple, sequential applications over time that accumulated to lethal concentrations were less lethal than one might expect based on concentration alone, opening the possibility that animals might be able to acclimate to the herbicide, although the underlying mechanism remains unclear. The present study also highlighted the ability of the herbicide to stratify in the water column, a phenomenon that has received very little attention, yet has important implications regarding both pesticide sampling on water bodies as well as how the habitat choices of individual organisms might make them more or less susceptible depending upon whether they move to strata that contain higher or lower pesticide concentrations. The implications of stratification are clear, but appear to be currently unexplored.

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