New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology

Rick A. Relyea[1]

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260 USA

Abstract.   The use of pesticides is important for growing crops and protecting human health by reducing the prevalence of targeted pest species. However, less attention is given to the potential unintended effects on nontarget species, including taxonomic groups that are of current conservation concern. One issue raised in recent years is the potential for pesticides to become more lethal in the presence of predatory cues, a phenomenon observed thus far only in the laboratory. A second issue is whether pesticides can induce unintended trait changes in nontarget species, particularly trait changes that might mimic adaptive responses to natural environmental stressors. Using outdoor mesocosms, I created simple wetland communities containing leaf litter, algae, zooplankton, and three species of tadpoles (wood frogs [*Rana sylvatica* or *Lithobates sylvaticus*], leopard frogs [*R. pipiens* or *L. pipiens*], and American toads [*Bufo americanus* or *Anaxyrus americanus*]). I exposed the communities to a factorial combination of environmentally relevant herbicide concentrations (0, 1, 2, or 3 mg acid equivalents [a.e.]/L of Roundup Original MAX) crossed with three predator-cue treatments (no predators, adult newts [*Notophthalmus viridescens*], or larval dragonflies [*Anax junius*]). Without predator cues, mortality rates from Roundup were consistent with past studies. Combined with cues from the most risky predator (i.e., dragonflies), Roundup became less lethal (in direct contrast to past laboratory studies). This reduction in mortality was likely caused by the herbicide stratifying in the water column and predator cues scaring the tadpoles down to the benthos where herbicide concentrations were lower. Even more striking was the discovery that Roundup induced morphological changes in the tadpoles. In wood frog and leopard frog tadpoles, Roundup induced relatively deeper tails in the same direction and of the same magnitude as the adaptive changes induced by dragonfly cues. To my knowledge, this is the first study to show that a pesticide can induce morphological changes in a vertebrate. Moreover, the data suggest that the herbicide might be activating the tadpoles' developmental pathways used for antipredator responses. Collectively, these discoveries suggest that the world's most widely applied herbicide may have much further-reaching effects on nontarget species than previous considered.

Key words: American toads (Bufo americanus *or* Anaxyrus americanus); *amphibian decline; dragonflies* (Anax junius); *glyphosate; inducible defense; leopard frogs* (Rana pipiens *or* Lithobates pipiens); *newts* (Notophthalmus viridescens); *phenotypic plasticity; synergy; wood frogs* (Rana sylvatica *or* Lithobates sylvaticus).

Introduction

To understand the impacts of anthropogenic chemicals on natural communities, we often base predictions on the plethora of single-species tests that are conducted as part of the pesticide regulation process. While a valuable first-step in determining the potential lethality of contaminants in nature over short periods (i.e., 1–4 d), these tests may tell us little about the impacts of contaminants on organisms under more natural conditions and over longer periods (Relyea and Hoverman 2006, Clements and Rohr 2009). Thus, many ecologists and toxicologists have moved to examine the impacts of contaminants under more natural conditions and over more realistic exposure times. In this manner, we can gain a better understanding of the direct and indirect effects of contaminants on nontarget organisms when embedded within their natural community.

Natural communities contain a number of potential factors that might interact with contaminants. Stressors, broadly defined as environmental factors that impair individual performance, can act either additively or synergistically. For contaminants, a number of abiotic and biotic stressors can make contaminants more or less lethal (Relyea and Hoverman 2006). Among biotic stressors, stress from low food or high competition can increase the lethality of contaminants (Postma et al. 1994, Hanazato and Hirokawa 2004, Beketov and Liess 2005, Jones et al. 2011). In addition, stress from the presence of predators can make several pesticides more lethal (Relyea and Mills 2001, Relyea 2003*b*, 2004*c*,

[1] E-mail: relyea@pitt.edu

2005*d*). However, synergistic interactions between pesticides and predatory stress have only been observed under laboratory conditions. We need to determine whether this lethal synergy occurs under more natural conditions.

While experiments have primarily examined whether natural stressors affect the lethality of contaminants, it is equally important to examine whether contaminants affect an organism's ability to adaptively respond to natural stressors. Any synergistic or antagonistic interactions of contaminants with stressors could cause organisms to become poorly suited to their environment. For example, biotic stressors such as competition and predation induce changes in the behavior, physiology, morphology, and life history of individuals (Miner et al. 2005). We have a good understanding of how contaminants alter the behavioral traits of target and nontarget animals, especially in the case of insecticides that commonly act on the animal's nervous system (reviewed in Weis et al. 2001). However, we know considerably less about the effects of contaminants on morphological traits, including those that are induced as a result of environmental stressors. For example, contaminants could interfere with an individual's normal environmental induction of morphology and cause the individual to develop a morphological phenotype that is poorly suited to the current environment. Such effects could alter morphological traits in ways that increase or decrease an individual's performance and ultimately its fitness. In some species of zooplankton, for example, insecticides can either induce morphological changes typically associated with predator defenses (Hanazato 1991, Barry 1998, 1999) or inhibit the induction of such traits (Hanazato 1999, Barry 1999, 2000). Beyond a few species of zooplankton, we have little information on the ability of contaminants to impact the induced morphological defenses of other taxonomic groups (but see Teplitsky et al. 2005).

I addressed these challenges using a mesocosm experiment in which I exposed an assemblage of three tadpole species to three levels of predatory stress, each in the presence of four concentrations of a globally common herbicide (glyphosate; commercially sold under many names including Roundup and Vision; Monsanto Corporation, St. Louis, Missouri, USA). Commercial formulations of glyphosate can be moderately to highly toxic to tadpoles under environmentally relevant concentrations (Bernal et al. 2009, Relyea and Jones 2009). Moreover, under laboratory conditions, the herbicide can be more lethal to tadpoles in the presence of waterborne cues emitted by predators (Relyea 2005*d*). To my knowledge, it is unknown whether predators can make the herbicide more lethal under mesocosm conditions nor whether the herbicide can induce morphological changes in any animal species. Tadpoles are an excellent model system in this regard because they are well known for expressing predator-induced changes in morphology including the development of relatively deeper tail fins (Van Buskirk 2002, Relyea 2003*b*, 2004*a*).

METHODS

Pesticide Background

Roundup Original MAX is one of numerous glyphosate-based herbicide formulations sold around the world by a variety of manufacturers. Collectively, glyphosate-based herbicides are the number one herbicide in the world with sales growing rapidly with the marketing of Roundup-Ready crops (Baylis 2000). Glyphosate products are used by homeowners, industry, and agriculture to kill undesirable plants. Glyphosate kills plants by preventing them from producing essential amino acids. For most plants, glyphosate alone has difficulty penetrating plant tissues due to the presence of the leaf cuticle layer, so a surfactant is typically added to introduce glyphosate into the plant. Polyethoxylated tallow amine (POEA) is one of the most commonly used surfactants. This surfactant can be highly toxic to fish and amphibians at application rates that are found in nature (Relyea 2006). While the surfactant of Roundup Original MAX is a trade secret (S. Mortenson, *personal communication*), the formulation has a toxicity to amphibians that is nearly identical to those formulations that are known to contain POEA (Relyea 2005*d*, Relyea and Jones 2009).

The concentrations of glyphosate-based herbicides in wetlands depend on whether the applications are inadvertent (e.g., applications over forests; Thompson et al. 2004) or due to drift, soil run-off, and plant wash-off. Expected worst-case concentrations, based on a range of assumptions regarding application rates, water depth, and interception by vegetation, range from 1.4 to 7.6 mg a.e./L (where a.e. stands for acid equivalents; Boutin et al. 1995, Mann and Bidwell 1999, Giesy et al. 2000, Solomon and Thompson 2003). Observed worst-case concentrations range from 1.7 to 5.2 mg a.e./L (Edwards et al. 1980, Giesy et al. 2000, Thompson et al. 2004). The half-life of glyphosate in pond water ranges from 8 to 120 d depending on environmental conditions (Barolo 1993).

The mesocosm experiment

The experiment employed a completely randomized design containing a factorial combination of four nominal Roundup concentrations (0, 1, 2, or 3 mg a.e./L of glyphosate) crossed with three predator treatments (no predator, caged adult newts [*Notophthalmus viridescens*], and caged larval dragonflies [*Anax junius*]). The resulting 12 treatment combinations were replicated four times for a total of 48 experimental units.

The experimental units were 757-L, outdoor mesocosms that contained many components of natural wetlands. The plastic mesocosms were filled with approximately 570 L of well water on 18–20 April 2006. On 21 April, I added 200 g of leaf litter (*Quercus* spp.) and 15 g of rabbit chow; both items serve as initial

RICK A. RELYEA

nutrient sources and the leaf litter also serves as a prey refuge. On the same day, I collected pond water from five ponds that contained zooplankton, phytoplankton, and periphyton. After screening the water for predators, I mixed water from all the ponds and added an aliquot of the resulting slurry to each tank. Soil was not added to the mesocosms because previous research has found that adding soil has no effect on the toxicity of Roundup under mesocosm conditions (Relyea 2005c). Each tank was covered with a 65% shade cloth lid to prevent other organisms from colonizing the tanks. Each tank also was equipped with four plastic lids (area = 45 cm^2) attached to a length of weighted plastic pipe and oriented vertically to serve as periphyton samplers.

Algal and zooplankton communities developed for 20 d, after which I added three species of larval amphibians. Amphibians were collected as newly oviposited eggs (wood frogs, *Rana sylvatica* [*Lithobates sylvaticus*] = 15 egg masses; northern leopard frogs, *R. pipiens* [*L. pipiens*] = 10 egg masses, American toads, *Bufo americanus* [*Anaxyrus americanus*] = 10 egg masses). The eggs were hatched and raised in wading pools containing aged well water and fed rabbit chow ad libitum. On 10 May (defined as day 0), I added 30 tadpoles of each species to every mesocosm for a total density of 90 tadpoles per mesocosm. This density of tadpoles (22 per m^2) is within the range of natural densities for these species during early ontogeny (R.A. Relyea, *unpublished data*). The tadpoles were early in development (approximately Gosner stage 25; Gosner 1960) with the following initial mean masses (±SE): wood frogs, 58 ± 4 mg; leopard frogs, 36 ± 1 mg; American toads, 16 ± 1 mg.

The predator treatments were added on day 1. Each mesocosm was equipped with two predator cages constructed from 236-mL plastic cups covered with a screen held on with a rubber band. In mesocosms assigned the no-predator treatment, the cages remained empty. In mesocosms assigned the newt or dragonfly treatments, each cage contained a single predator that was fed approximately 300 mg of tadpoles (a mixture of wood frogs and leopard frogs) three times per week. On each feeding day, I also lifted the empty cages in the no-predator treatment to equalize disturbance across all tanks.

The herbicide treatments were applied on day 2. I used a popular formulation of glyphosate (Roundup Original MAX; Monsanto Corporation, St. Louis, Missouri, USA) that contained 540 mg a.e./L of glyphosate plus an undisclosed surfactant. To attain the nominal concentrations of 0, 1, 2, and 3 mg a.e./L of glyphosate, I added 0, 0.864, 1.728, and 2.592 mg of formulated product to the mesocosms, respectively. The formulated product was added to 300 mL well water and then this mixture was distributed evenly across the surface of the mesocosms. One hour after the applications, I sampled the water of all mesocosms in the middle of the water column and pooled the samples by herbicide treatment. The water samples were then frozen and later shipped to the Mississippi State Chemical Lab (Mississippi State, Mississippi, USA) for analysis using high-pressure liquid chromatography. The analyses indicated that the actual concentrations were 0.9, 1.8, and 3.4 mg a.e./L. Given that these values are within 13% of the nominal values with no directional bias, the concentrations will hereafter be referred to as 1, 2, and 3 mg a.e./L.

On the afternoon of day 9, I measured temperature, pH, and dissolved oxygen of all mesocosms. Measurements were taken with a calibrated, digital water meter. Across all treatments, there was little variation in pH (range, 7.8–8.0) or temperature (range, 11.7–11.9°C). In contrast, dissolved oxygen varied widely among treatments (range, 10–20 mg/L), so I analyzed the treatment effects on dissolved oxygen.

On days 15 and 21, I sampled the periphyton in each mesocosm. On each date, I removed two periphyton sampler discs from the same location in each mesocosm, brushed them free of periphyton, and then rinsed the samplers with well water. The slurry of periphyton and water was filtered through pre-dried and pre-weighed Whatman GF/C filters. After filtering the periphyton slurry, the filters were dried for 24 h at 80°C and re-weighed to estimate periphyton availability in each mesocosm.

On day 21, I terminated the experiment by removing all water and leaf litter and recovering all surviving tadpoles. The survivors were euthanized in 2% MS-222 and then preserved in 10% formalin. The preserved animals were later counted and weighed to determine survival and average individual mass for each species. The amphibian response variables were the proportion of each species surviving in each mesocosm and the mean individual mass of each species in each mesocosm.

Morphological measurements

One of the objectives of this study was to examine whether exposure to the herbicide affected tadpole morphology. Because a large proportion of tadpoles died in the highest herbicide treatment, I excluded this treatment from the morphological analyses. Two of the three species (wood frogs and leopard frogs) possess highly plastic morphological responses to predators and competitors (Relyea and Werner 1999, Relyea 2003a, Schoeppner and Relyea 2009), so only these two species were assessed for predator- and herbicide-induced changes in relative morphology. Past studies on American toads suggests that they are less plastic (Relyea 2001). I measured seven morphological traits on each surviving tadpole: tail depth and length, body depth, length, and width, and tail muscle depth and width (see Fig. 1 in Relyea 2000).

Statistical analyses

I analyzed the data using analyses of variance. I conducted separate multivariate analyses of variance

TABLE 1. Multivariate and univariate test results examining the effects of three predator cue treatments crossed with four concentrations of glyphosphate (as the commercial formulation Roundup Original MAX) on the survival of wood frog, leopard frog, and American toad tadpoles.

	Multivariate test (Wilks' lambda)			Univariate tests (P)			
Treatment	df	F	P	df	Wood frog survival	Leopard frog survival	Toad survival
Herbicide	9,108	7.2	<0.001	3,36	<0.001	<0.001	<0.001
Predator	6,70	2.6	0.027	2,36	0.047	0.077	0.068
Herbicide × Predator	18,108	1.6	0.063	6,36	0.010	0.068	0.618

Note: In the univariate tests, only df and P values are reported.

(MANOVAs) on survival and mass, because some mesocosms at the highest glyphosate concentration had no surviving animals to permit an assessment of mass. The survival data for wood frogs and leopard frogs were heteroscedastic and the data for leopard frogs marginally non-normal. The data distribution could not be improved by any transformation, but analyses of variance are robust to such violations when the data are balanced and independent (Quinn and Keough 2002), particularly when the largest variance is not more than four times larger than the smallest variance (which was the case for leopard frog survival but not for wood frog survival). The mass data were both homoscedastic and normally distributed. The analysis of mass did not include survival as a covariate because there was no indication that treatments that caused tadpole mortality were associated with increased individual mass due to reduced competition.

To estimate the LC50 values (i.e., the concentration required to kill 50% of a population) for each species under each predator treatment, I conducted probit analyses. Using 84% confidence intervals for these estimates allows one to determine differences among predator treatments because non-overlapping 84% confidence intervals are significant at approximately α = 0.05 (Payton et al. 2003).

The next set of analyses examined the effects of the treatments on tadpole morphology. Because linear morphological measurements are typically larger on animals of larger mass, I examined mass-adjusted morphological traits. To do this, I conducted a MANCOVA on all seven log-transformed linear dimensions and included log-transformed mass as a covariate and the two treatments (predators and pesticides) as fixed effects. For most traits in both species, I was able to confirm that there were no significant two- or three-way mass-by-treatment interactions (a key assumption in mass-adjustment analyses). However, there was a significant multivariate mass-by-predator interaction in wood frogs. Examination of the univariate responses indicated that there was an interaction of mass and predators effect on tail depth; whereas the smallest animals had tail depths of similar size, the magnitude of predator induction on tail depth continually increased as tadpole mass increased. This is not surprising given that

the ability to express a plastic response in morphology increases with increased growth (Relyea 2004b). Ignoring this mass-by-predator interaction would estimate the magnitude of predator induction for a tadpole of average mass. This overestimates predator induction of the smallest tadpoles and underestimates predator induction of the largest tadpoles, but provides an unbiased estimate of predator induction for average sized tadpoles. In contrast, there were no significant mass-by-herbicide interactions on tadpole morphology, so any herbicide induction of tadpole morphology could be estimated without bias.

From the MANCOVA, I saved the residuals from each tadpole and the estimated marginal means for each treatment. I added these two values to determine the mass-adjusted morphology of each tadpole. For wood frog and leopard tadpoles, I then calculated mesocosm means, which served as response variables.

The mass-adjusted morphological traits of the leopard frogs and wood frogs were analyzed using separate MANOVAs. The leopard frog data had homoscedastic errors and were normally distributed. The wood frog data had homoscedastic errors for all of the morphological traits except for muscle width and all data were normally distributed. Significant multivariate effects were followed by univariate tests and pairwise comparisons (Tukey's hsd).

I also analyzed treatment effects on periphyton biomass and dissolved oxygen concentrations. Periphyton biomass was analyzed using a repeated-measures analyses of variance (rm-ANOVA). The log-transformed data were normally distributed and the errors were either homoscedastic or marginally homoscedastic. Dissolved oxygen data were analyzed using an ANOVA. Assumptions of normality and homoscedasticity were both met. All mean comparisons were conducted using Tukey's hsd.

RESULTS

Tadpole survival

There were multivariate effects of herbicide and predators and a marginal concentration-by-predator interaction (Table 1, Fig. 1). The subsequent ANOVAs illuminated which species were driving these effects.
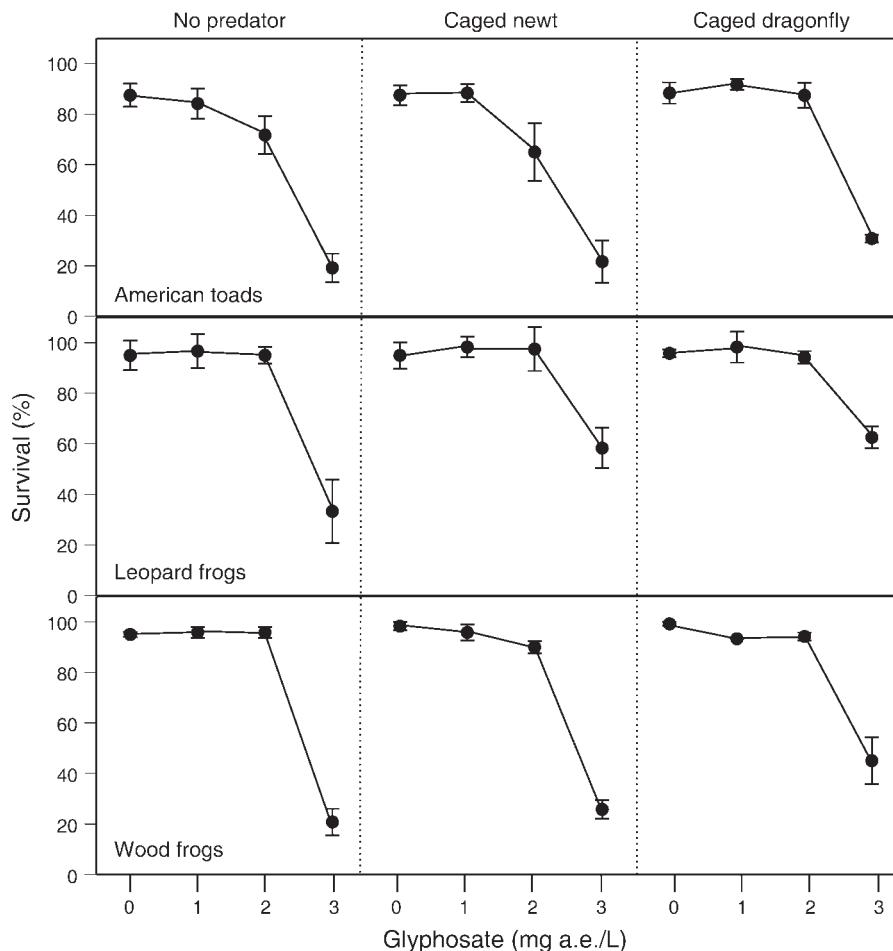
Fig. 1. The survival of three species of tadpoles when exposed to a factorial combination of caged-predator treatments crossed with four concentrations of glyphosphate (as the commercial formulation Roundup Original MAX). Data are means ± SE; "a.e." stands for acid equivalents.

American toad survival was affected by the herbicide and there was a marginal effect of predators, but no herbicide-by-predator interaction (Table 1, Fig.1). Toads in the controls averaged 88% survival and toads exposed to 1 mg a.e./L exhibited no reduction in survival ($P = 0.955$). In contrast, toads exposed to 2 mg a.e./L experienced an average survival of 75% ($P = 0.011$) and toads exposed to 3 mg a.e./L experienced an average survival of 24% ($P < 0.001$). Although the herbicide-by-predator interaction was not significant, it is clear from the data that the marginally significant predator effect was driven by glyphosate's lethal effects being reduced in the presence of caged dragonflies. For example, at 2 mg a.e./L, survival was reduced by 16% with no predators but was only reduced by 1% with caged dragonflies. Similarly, at 3 mg a.e./L, survival was reduced by 68% with no predators but was only reduced by 58% with caged dragonflies. Based on the probit analyses, the LC50 for toads ranged from 2.4 to 2.8 mg a.e./L (Table 2).

TABLE 2. Estimated LC50 values (i.e., the concentration required to kill 50% of a population) for three species of tadpoles when exposed to a range of concentrations of glyphosphate (as the commercial formulation Roundup Original MAX) in the presence of three caged-predator environments.

Species	Caged predator	LC50	84% CI
Wood frog	no predator	2.95	-
	caged newt	2.63	2.45, 2.84
	caged dragonfly	3.09	2.75, 3.80
Leopard frog	no predator	2.91	2.74, 3.03
	caged newt	3.02	-
	caged dragonfly	3.26	3.02, 3.90
American toad	no predator	2.46	2.27, 2.69
	caged newt	2.44	2.26, 2.64
	caged dragonfly	2.82	2.66, 2.96

Notes: Estimates are followed by 84% confidence intervals; nonoverlapping confidence intervals are significant at approximately $\alpha = 0.05$ (Payton et al. 2003). In two cases, indicated by dashes, the confidence interval could not be estimated due to the distribution of the data.

Leopard frog survival was affected by the herbicide and marginally affected by predators and the concentration-by-predator interaction (Table 1, Fig.1). Once again, the interaction occurred because the impact of the herbicide was larger without predators than with caged dragonflies. Increasing the herbicide from 0 to 3 mg a.e./L caused 62% death without predators, but only 37% death with caged newts and 33% death with caged dragonflies (all $P \leq 0.002$). Based on the probit analyses, the LC50 ranged from 2.9 to 3.2 mg a.e./L (Table 2).

Wood frog survival was affected by herbicide, predator, and an herbicide-by-predator interaction (Table 1, Fig.1). The interaction occurred because increasing the herbicide concentration from 0 to 3 mg a.e./L caused 74% death without predators and 73% death with caged newts, but caused only 54% death with caged dragonflies (all $P < 0.001$). Based on the probit analyses, the LC50 for wood frogs ranged from 2.6 to 3.1 mg a.e./L (Table 2).

Tadpole mass

Tadpole mass exhibited significant effects of herbicide, predators, and the herbicide-by-predator interaction (Table 3, Fig. 2). Subsequent ANOVAs determined which of the three tadpole species was driving these multivariate effects. American toad mass exhibited no effects of glyphosate or predators (Table 3, Fig. 2).

Leopard frog mass exhibited a main effect of herbicide, no main effect of predators, and a significant interaction (Table 3, Fig. 2). Without predators, there was no effect of the herbicide ($P > 0.3$). With caged newts, there also was no effect of the herbicide ($P > 0.08$). With caged dragonflies, however, there was no effect of 1 or 2 mg a.e./L ($P > 0.7$) compared to the control, but there was a large (37%) decline in mass with 3 mg a.e./L ($P = 0.001$).

Wood frog mass was affected by herbicide, predators, and their interaction (Table 3, Fig. 2). To understand the nature of the interaction, I examined how increased herbicide concentrations affected growth compared to the control within each predator treatment. When predators were absent, there was no effect of 1 mg a.e./L ($P = 0.434$) compared to the control, a marginal decline in mass (13%) with 2 mg a.e./L ($P = 0.070$), and a significant decline in mass (15%) with 3 mg a.e./L ($P = 0.032$). With caged newts, there was no effect of 1 or 2 mg a.e./L ($P > 0.3$) compared to the control, but there was a marginal decline in mass (17%) with 3 mg a.e./L ($P = 0.054$). With caged dragonflies, there was no effect of 1 or 2 mg a.e./L ($P > 0.9$) compared to the control, but there was a large (38%) decline in mass with 3 mg a.e./L ($P < 0.001$).

Tadpole morphology

Leopard frogs.—The analysis of leopard frog morphology found significant multivariate effects of the herbicide and predator treatments, but no herbicide-by-predator interaction (Fig. 3, Table 4). Univariate tests indicated that these effects were caused by herbicide and predator effects on tail depth, body depth, and body length (Table 4).

Tail depth was affected by both the predator and herbicide treatments (Table 4). Compared to the no-predator control, caged newts induced no change ($P = 0.176$) while caged dragonflies induced deeper tails ($P = 0.004$). Compared to the herbicide control, 1 mg a.e./L had no effect on tail depth ($P = 0.984$) while 2 mg a.e./L induced deeper tails ($P = 0.006$).

Body depth was affected by the herbicide treatments but not by the predator treatments (Table 4). There was no difference between 0 mg a.e./L and the other two concentrations (both $P > 0.2$), but bodies were deeper with 2 mg a.e./L than with 1 mg a.e./L ($P = 0.014$).

Body length was affected by the predator treatments but not by the herbicide treatments (Table 4). Compared to the no-predator treatment, newts induced similar body lengths ($P = 0.677$) while dragonflies induced shorter body lengths ($P = 0.044$).

In summary, caged dragonflies induced leopard frogs to develop deeper tails and shorter bodies than the no-predator control. Interestingly, 2 mg a.e./L of the herbicide also induced deeper tails as well as changes in body depth.

Wood frogs.—The analysis of wood frog morphology found significant multivariate effects of the herbicide and predator treatments, but no herbicide-by-predator interaction (Fig. 3, Table 5). Univariate tests indicated that the treatments affected four morphological traits: muscle depth, tail depth, body depth, and body length.

The muscle depth of wood frogs was only affected by the herbicide treatments (Table 5). Compared to 0 mg a.e./L, 1 mg a.e./L had no effect ($P = 0.933$) but 2 mg a.e./L induced deeper tail muscles ($P = 0.009$).

Wood frog tail depth was affected by both the predator and the herbicide treatments (Table 5). Compared to the no-predator control, caged newts had no effect ($P = 0.123$) and caged dragonflies induced significantly deeper tails ($P < 0.001$). Compared to 0 mg a.e./L, 1 mg a.e./L had no effect ($P = 0.990$) but 2 mg a.e./L induced deeper tails ($P = 0.011$).

Body depth was marginally affected by the herbicide but not by predators (Table 5). Bodies exposed to 0 mg a.e./L tended to be deeper than 1 mg a.e./L ($P = 0.057$) but not different from 2 mg a.e./L ($P = 0.661$). The latter two concentrations did not differ ($P = 0.288$).

Body length was affected by the predator treatments but not by the herbicide treatments (Table 5). Compared to the no-predator control, body length was marginally reduced by caged newts ($P = 0.081$) and significantly reduced by caged dragonflies ($P = 0.005$).

In summary, caged dragonflies induced wood frogs to develop deeper tails and shorter bodies than the no-predator control. An exposure to 2 mg a.e./L of the herbicide induced deeper tails and tail muscles than 0 mg a.e./L. The herbicide also induced changes in body depth.

RICK A. RELYEA

TABLE 3. Multivariate and univariate test results examining the effects of three predator cue treatments crossed with four concentrations of glyphosphate (as the commercial formulation Roundup Original MAX) on the mass of wood frog, leopard frog, and American toad tadpoles.

| | Multivariate test (Wilks' lambda) | | | Univariate tests (P) | | | |
Treatment	df	F	P	df	Wood frog mass	Leopard frog mass	Toad mass
Herbicide	9,105	4.4	<0.001	2,27	<0.001	0.002	0.508
Predator	6,68	2.5	0.030	2,27	0.002	0.146	0.622
Herbicide × Predator	18,105	2.5	0.002	4,27	0.002	0.014	0.117

Note: In the univariate tests, only df and P values are reported.

Periphyton and dissolved oxygen

The analysis of periphyton found an effect of herbicide ($F_{3,34} = 4.3$, $P = 0.012$) and time ($F_{1,34} = 18.3$, $P < 0.001$), but no predator effect ($F_{2,34} = 0.05$, $P = 0.952$) or any interactions (all $P > 0.17$; Fig. 4). Periphyton declined over time, but averaged over time and across all predator treatments, periphyton was similarly abundant between the control and either 1 or 2 mg a.e./L ($P > 0.9$), but was 83% more abundant with 3 mg a.e./L ($P = 0.039$).

The ANOVA on dissolved oxygen found a significant effect of herbicide ($F_{3,36} = 14.4$, $P < 0.001$), but no effect of predator ($F_{2,36} = 0.2$, $P = 0.797$) or an herbicide-by-predator interaction ($F_{6,36} = 0.6$, $P = 0.707$; Fig. 4). Averaged across predators, dissolved oxygen was highest in the absence of the herbicide. Compared to
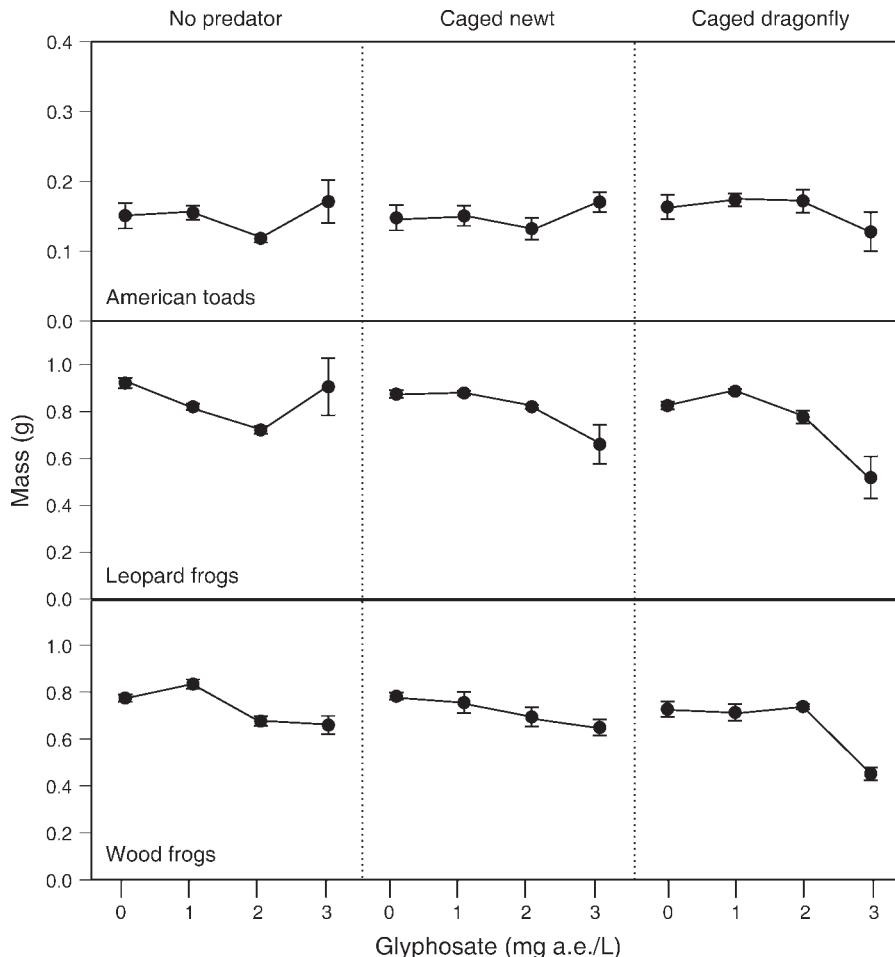

FIG. 2. The mass of three species of tadpoles when exposed to a factorial combination of caged-predator treatments crossed with three concentrations of glyphosphate (as the commercial formulation Roundup Original MAX). The highest nominal concentration used in the experiment (3 mg a.e./L) resulted in too few survivors to reliably assess tadpole mass. Data are means ± SE.
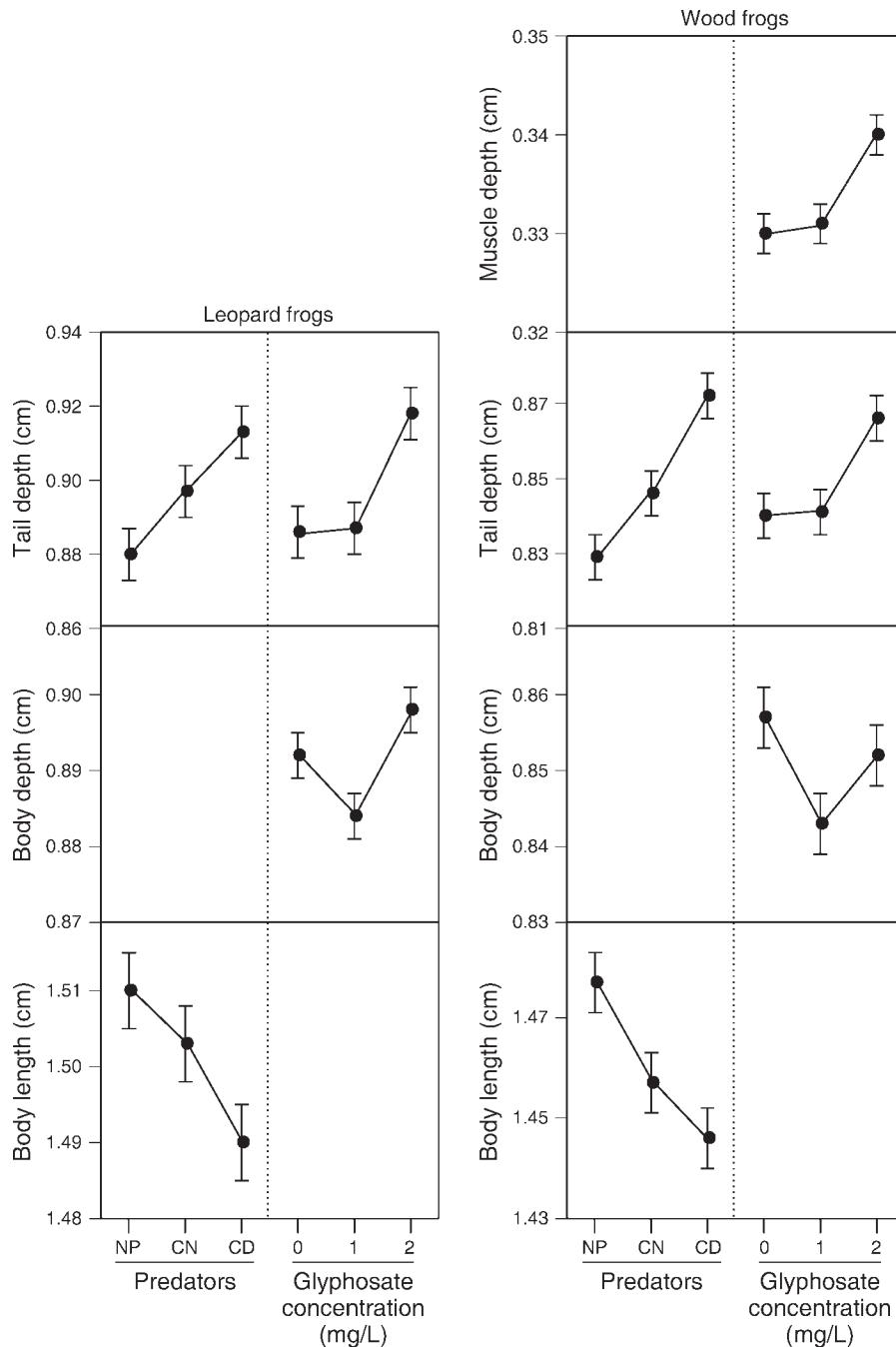
FIG. 3. The relative morphology of leopard frog and wood frog tadpoles when exposed to a factorial combination of caged-predator treatments crossed with three concentrations of glyphosate (as the commercial formulation Roundup Original MAX). Because the predator and herbicide treatments did not interact, the displayed predator effects are averaged across herbicide treatments, and the displayed herbicide effects are averaged predator treatments. The highest nominal concentration used in the experiment (3 mg a.e./L) resulted in too few survivors to assess morphology. Data are means ± SE. Only significant main effects are shown.

the control, additions of 1, 2, or 3 mg a.e./L caused 28 to 35% reductions in dissolved oxygen (all $P < 0.001$). The latter three treatments did not differ from each other (all $P > 0.6$). Across all herbicide concentrations, dissolved oxygen concentrations remained quite high.

DISCUSSION

The experiment demonstrated that glyphosate-based herbicides and predator cues can have surprising effects on amphibians and wetland communities. Environmentally relevant concentrations of the herbicide caused

TABLE 4. Multivariate and univariate test results examining the effects of three predator cue treatments crossed with three concentrations of glyphosphate (as the commercial formulation Roundup Original MAX) on the relative morphology of leopard frog tadpoles.

	Multivariate test (Wilks' lambda)			Univariate tests (P)							
					Tail		Body			Muscle	
Treatment	df	F	P	df	Depth	Length	Depth	Length	Width	Depth	Width
Herbicide	14,42	2.9	0.003	2,27	0.003	0.807	0.019	0.329	0.457	0.224	0.441
Predator	14,42	2.3	0.021	2,27	0.006	0.863	0.986	0.050	0.996	0.826	0.113
Herbicide × Predator	28,77	1.3	0.169								

Note: In the univariate tests, only df and P values are reported.

high rates of mortality in three species of tadpoles, reductions in growth for two of the three species (i.e., wood frogs and leopard frogs), and several indirect effects including an increase in periphyton and a decrease in dissolved oxygen (although oxygen concentrations were always well above those that would cause any harmful effects). When the different concentrations were crossed with the chemical cues emitted by predators, the herbicide exposures became less lethal which is in direct contrast to lab studies that have found that predator cues can make the herbicide more lethal. As in past studies, the tadpoles responded to the highest level of predatory stress (i.e., caged dragonflies) by altering their morphology in adaptive directions. Unexpectedly, however, the herbicide also induced changes in the tadpole's morphology with a direction and magnitude that were nearly identical to the morphological changes induced by the caged dragonflies. To my knowledge, this is the first example of pesticide-induced morphological plasticity in amphibians or any other vertebrate.

Predators facilitate tadpole survival when exposed to herbicides

The mortality caused by the herbicide was within the range observed in past experiments. Across the three species and the three predator environments, LC50 values ranged from 2.4 to 3.3 mg a.e./L. A large number of laboratory studies have been conducted on commercial formulations of the herbicide containing either the POEA surfactant or an undisclosed surfactant possessing a toxicity similar to POEA (e.g., Roundup Original, Roundup Original MAX, Roundup Weathermax, Vision, Cosmo-Flux). These lab studies, which typically change the water and reapply the pesticide every 1–4 d, have found LC50 values ranging from 0.4 to 11.6 mg a.e./L (Mann and Bidwell 1999, Lajmanovich et al. 2003, Edginton et al. 2004, Howe et al. 2004, Relyea 2005d, Bernal et al. 2009, Relyea and Jones 2009, Dinehart et al. 2010). Based on the standard toxicity definitions used by the U.S. Environmental Protection Agency, these commercial formulations range from slightly toxic (10 mg/L < LC50 < 100 mg/L) to highly toxic (0.1 mg/L < LC50 < 1 mg/L; toxicity definitions *available online*).[2]

A growing number of toxicity studies have been conducted under mesocosm conditions and have observed effects that are consistent with the laboratory studies. For example, Relyea (2005b) found that 3 mg a.e./L caused high rates of tadpole mortality, with several species including wood frogs, leopard frogs, American toads, and gray tree frogs (*Hyla versicolor*) being nearly or completely eliminated. A follow-up study tested whether the addition of sand or loam soil would ameliorate the effects of Roundup on three species of tadpoles (leopard frogs, American toads, and gray tree frogs). In that study, neither sand nor loam reduced the high rates of tadpole mortality (Relyea 2005c). Using one-third as much Roundup (1 mg a.e./L), Relyea et al. (2005) found no effect on gray tree frog tadpoles, but observed 29% death in leopard frogs and 71% in American toads. As in the current experiment, all of these mesocosm experiments were conducted using well water that had an approximate pH = 8. More recently, Jones et al. (2010) observed similar high mortality levels in wood frog and American toad tadpoles exposed to Roundup concentrations up to 3 mg a.e./L, but found that applications later in ontogeny were less lethal than applications earlier in ontogeny. Using a different suite of species (American bullfrogs [*R. catesbeiana*], green frogs [*R. clamitans*], and gray tree frogs), Jones et al. (2011) found that the high rates of mortality at 2 and 3 mg a.e./L can be further increased under conditions of increased competition. Importantly, experiments conducted at lower pH values have found lower rates of mortality at similar concentrations of glyphosate + POEA (Wojtaszek et al. 2004). As noted by past authors, this means that the toxicity of these herbicide formulations are of particular concern in wetlands on the upper end of the naturally occurring pH range (Chen et al. 2004, Edginton et al. 2004).

As reviewed by Relyea (2011), a key issue in evaluating the potential impact of glyphosate-based herbicides is to consider the concentrations found in nature. Unfortunately, there is a paucity of data on the

[2] http://www.epa.gov/espp/litstatus/effects/redleg-frog/

TABLE 5. Multivariate and univariate test results examining the effects of three predator cue treatments crossed with three concentrations of glyphosphate (as the commercial formulation Roundup Original MAX) on the relative morphology of wood frog tadpoles.

| | Multivariate test (Wilks' lambda) | | | Univariate tests (P) | | | | | | | |
| | | | | | Tail | | Body | | | Muscle | |
Treatment	df	F	P	df	Depth	Length	Depth	Length	Width	Depth	Width
Herbicide	14,42	2.8	0.005	2,27	0.005	0.178	0.066	0.288	0.650	0.006	0.903
Predator	14,42	3.1	0.002	2,27	<0.001	0.949	0.323	0.006	0.802	0.326	0.255
Herbicide × Predator	28,77	1.0	0.463								

Note: In the univariate tests, only df and P values are reported.

concentrations of glyphosate in natural ponds and wetlands because most major surveys of pesticides in water bodies have been restricted to lakes, streams, and rivers. In contrast, most amphibians do not live in these habitats. For ponds and wetlands the expected worst-case scenarios for terrestrial formulations of glyphosate (which are generally not recommended for aquatic applications) range from 1.4 to 7.6 mg a.e./L depending on the assumptions used (Boutin et al. 1995, Mann and Bidwell 1999, Giesy et al. 2000, Solomon and Thompson 2003). Actual worst-case scenarios range from 1.7 to 5.2 mg a.e./L (Edwards et al. 1980, Giesy et al. 2000, Thompson et al. 2004). Mean concentrations across a landscape of inadvertently oversprayed wetlands can be considerably lower e.g., 0.33 mg a.e./L; Thompson et al. 2004). However, it is important to realize that a given pond does not experience the mean concentration, but instead receives a specific concentration. For example, while Thompson et al. (2004) found that the mean concentration in forested wetlands (after spraying to favor conifer trees over broadleaf trees) was 0.33 mg a.e./L, they found that individual wetlands had up to 1.95 mg a.e./L, a concentration that can cause high amphibian mortality.

While the high toxicity of this globally common herbicide has been well documented under both lab and mesocosm conditions, the observation that predator cues can make the herbicide less lethal under mesocosm conditions is a novel discovery. The most likely explanation for this observation is herbicide stratification. Recent studies have discovered that the water column can experience thermal stratification which, in turn, causes glyphosate-based herbicides to be two to four times more concentrated near the surface than near the benthos (Jones et al. 2010, 2011). Although the current experiment sampled the water in the middle of the water column rather than separately sampling near the surface and near the benthos, it is reasonable to assume that the mesocosms in the current study also experienced stratification, given that the current study and Jones et al. (2010) were conducted in the same location, during similar times of year, under similar conditions, and using the same formulation of Roundup. Because the stratification of lentic habitats is a common phenomenon, it is not surprising in hindsight that pesticides can stratify. Indeed, pesticide stratifica-

tion is a phenomenon that has been observed in natural wetlands in at least two studies (Sudo et al. 2004, Ma et al. 2008). The frequency of such stratification will depend on ambient temperatures, pond depth, and the existence of winds that can potentially cause a stratified water column to mix. What is interesting is that the stratification of Roundup sets the stage for the interactive effects of predator cues and herbicide concentrations.

The current study included three predator treatments that represented increasing levels of predation risk to tadpoles (Babbitt 2001, Relyea 2003a) that typically induce different magnitudes of antipredator responses. When predator cages are positioned at the surface,
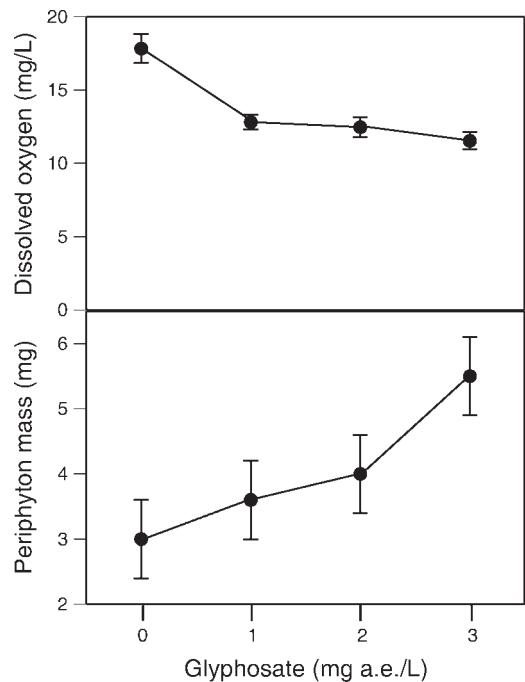

FIG. 4. The biomass of periphyton (averaged across samples taken on day 15 and 21) and the dissolved concentration of oxygen (on day 9) in mesocosms exposed to a factorial combination of caged-predator treatments crossed with four concentrations of glyphosphate (as the commercial formulation Roundup Original MAX). Periphyton was sampled on two dates. Samples are based on two 45-cm² samplers per mesocosm on each date. Data are means ± SE.

tadpoles typically move down to the benthos (Relyea 2001, Schoeppner and Relyea 2009) and this behavior was noted in the current study (R. A. Relyea, *personal observation*). This move to the benthos would have placed the tadpoles in a region of the water column that had a lower herbicide concentration and, as a result, fewer tadpoles died. In short, herbicide stratification and predator-induced changes in the habitat use of tadpoles combined to reduce the amount of herbicide-caused mortality by 20% in wood frogs, 29% in leopard frogs, and 10% in toads. The smaller effect in toads might be due to the caged predators being fed only tadpoles of wood frogs and leopard frogs; past studies have shown that tadpoles exhibit small differences in their antipredator traits when predators consume tadpole diets that span different families (Schoeppner and Relyea 2005). Given that predator avoidance is common in a wide range of aquatic taxa (Lima 2002), it is possible that predator cues and the stratification of contaminants could cause interactive effects in other taxonomic groups as well.

To my knowledge, the only experimental assessment of predatory stress and glyphosate-based herbicides was the lab experiment of Relyea (2005d) which tested six species of tadpoles in 8-L tubs of water and found that one of the species (wood frogs) experienced significantly *higher* rates of mortality when the herbicide was combined with predatory cues emitted by caged newts (in that study, leopard frogs and American toads exhibited no synergistic interactions). In the current experiment, the synergy was observed for wood frogs and leopards (significant for wood frogs, marginally nonsignificant for leopard frogs), but in the *opposite* direction. However, the density of caged newts in the lab experiments (one newt in 8 L of water) was much higher than the density of caged newts in the current mesocosm experiment (four newts in 570 L of water). Although each newt in the mesocosm experiment was also fed three times more tadpole biomass per day than the lab experiments, the concentration of predator cues in the earlier lab experiments was still nearly six times more concentrated than the current mesocosm experiment. Assuming increased concentrations of predator cue lead to stronger synergistic interactions with pesticides, this difference should make it less likely that predator cues would increase the lethality of the herbicide in the mesocosm experiment. Importantly, however, this difference in predator cue concentration does not explain the observation that predator cues actually *decreased* the lethality of the herbicide in the mesocosm experiment.

Tadpole mass and periphyton abundance

Changes in tadpole survival were concomitant with herbicide and predator effects on tadpole mass in two of the three species (i.e., wood frogs and leopard frogs). In general, the strongest reductions in tadpole mass occurred when tadpoles were exposed to the most risky predator (i.e., dragonflies) combined with the highest concentration of the herbicide. Because the periphyton was more abundant under the highest herbicide concentration, due to fewer surviving tadpoles, the reduced growth of the tadpoles was not due to the herbicide inhibiting periphyton growth. Indeed, under lab conditions in which tadpoles were fed a constant per-capita food ration, many species of tadpoles grew slower when exposed to 1.5 mg a.e./L of glyphosate + POEA (Relyea 2004b). Under laboratory conditions, therefore, it seems that the herbicide can affect the ability of tadpoles to consume their food or convert their food into growth. Under mesocosm conditions, however, a reduction in tadpole growth has not been previously observed. For example, herbicide applications of 1 to 3 mg a.e./L (similar to the no-predator treatments in the current experiment) early in tadpole development had no effect on tadpole growth (Jones et al. 2010). In another experiment that manipulated herbicide concentration and tadpole competition, Jones et al. (2011) found that tadpole growth actually increased with higher herbicide concentrations. The key observation in the current experiment is that the decline in tadpole mass only occurred when cues from the most dangerous predator (which induces reduced foraging activity; Relyea 2002b, 2003a) were combined with the highest concentration of the pesticide. In short, it appears that a reduction in tadpole mass requires both stressors to be present. Reductions in mass are important to amphibians because reduced mass at metamorphosis is associated with reduced post-metamorphic survival, longer times to reproductive maturity, reduced size at maturity, reduced mating success, and smaller clutches of eggs (Smith 1987, Semlitsch et al. 1988, Altwegg and Reyer 2003).

The sharp decline in tadpole survival caused by the highest concentrations of Roundup was the most likely cause of the increased standing crop of periphyton (i.e., the food source of tadpoles). If the herbicide had negative direct effects on periphyton, they were more than overcome by the indirect positive effects of removing the tadpole grazers. Such an outcome would be expected whenever consumers are food limited, as in the current experiment in which a total of 90 tadpoles were added to each mesocosm. A similar increase in periphyton was observed in a previous mesocosm experiment that was initiated with a high density of periphyton consumers (i.e., 50 tadpoles and 30 snails). In that experiment, adding 3 mg a.e./L of Roundup caused high rates of tadpole mortality and a concomitant increase in periphyton (Relyea 2005b). Similarly, an experiment using 60–140 tadpoles per mesocosm found that adding 1–3 mg a.e./L of the herbicide caused an increase in periphyton biomass (Jones et al. 2011). In contrast, a mesocosm experiment that was initiated with 40 tadpoles found no effects on periphyton biomass (Jones et al. 2010). Collectively, these studies suggest that herbicide-caused increases in periphyton are more likely when there is more intense competition among tadpoles.

Given that higher concentrations of the herbicide were associated with increases in periphyton, it is perhaps surprising that there was a decline in the concentration of dissolved oxygen for all three herbicide additions (although dissolved oxygen never approached a level that would negatively impact the tadpoles). Increased microbial decomposition of dead tadpoles in the 3 mg a.e./L compared to 1 mg a.e./L treatment does not explain this pattern because dissolved oxygen was similar in these two treatments. Instead, it seems more likely that the herbicide may have been inhibiting the growth of the phytoplankton in the water column. Indeed, past studies have shown that Roundup can cause declines in phytoplankton (Perez et al. 2007), with LC50 values ranging from 1.9 to 5.8 mg a.e./L (Tsui and Chi 2003). Because phytoplankton was not measured in this experiment, the hypothesized mechanism should be examined in future work.

The induction of tadpole morphology

Leopard frogs and wood frogs exhibited changes in relative morphology when exposed to predators and the herbicide. Predator-induced morphology is taxonomically widespread and appears to be an adaptive response to reduce the tadpole's risk of being killed by predators (Van Buskirk 2002, Relyea 2003a, 2004a, 2005a). In most cases, tadpoles respond to predators by building a relatively deep tail fin, which helps evade deadly predator strikes, at the cost of building a relatively smaller body, which leads to slower growth likely due to reduced food consumption and reduced digestive efficiency (Relyea 2000, Relyea and Auld 2004, 2005). As in the case of behavioral responses, tadpoles typically exhibit stronger morphological responses to the most risky predators. Not surprisingly, the current study found that both wood frog and leopard frog tadpoles exhibited weak morphological induction in response to the less risky newts but strong morphological induction in response to dragonflies.

Surprisingly, the herbicide-induced changes in tadpole morphology and did so in the same direction and with the same magnitude as the caged dragonflies. The change in tail depth was particularly striking; for leopard frogs and wood frogs, respectively, the addition of dragonfly chemical cues caused a 3.8% and 5.2% increase in tail depth and the addition of Roundup induced a 3.6% and 3.1% increase in tail depth. It is also interesting that the two factors had additive effects on tail depth, rather than synergistic or antagonistic effects. The combination of dragonfly cues and the herbicide (at 2 mg a.e./L) induced a 6.7–9.3% increase in tail depth for leopard frogs and wood frogs, respectively; this was approximately twice as large as either factor induced alone. The fitness consequences of herbicide-induced plasticity is unknown. However, based on past studies of predator-induced morphology, one would predict that this herbicide induction would have an associated cost of slower growth (Relyea 2002a), but this was not observed within the 0 to 2 mg a.e./L range of concentrations that caused morphological induction. Although the mechanism underlying the ability of Roundup to induce morphological changes in tadpoles is unknown, it is a reasonable hypothesis that the herbicide may be interfering with the stress hormones that induce antipredator defenses (Glennemeier and Denver 2002).

Examples of a pesticide inducing morphological changes in animals are rare and limited to a few species of cladocerans. Nearly two decades ago, Hanazato (1991) reported that several organophosphate and carbamate insecticides induced morphological changes in Daphnia ambigua that resembled the same morphological changes induced by predators. Two more recent studies have found insecticides can induce elongated crests, a predator-induced phenotype, in Daphnia (Barry 1998, Oda et al. 2011). On the other hand, studies on Bosmina fatalis have found that insecticides can impede the induction of predator-induced morphology (Barry 1999, 2000, Sakamoto et al. 2006). There appear to be no studies examining the effects of herbicides on the defensive morphology of cladocerans.

Conclusions

The results of this study demonstrate that the impact of the most widely applied herbicide in the world has a number of unexpected effects that often interact with the common natural stressor of predator cues. The high rates of mortality demonstrated in previous studies at environmentally relevant concentrations were fully supported in the current study, further confirming that glyphosate-based herbicides containing the POEA surfactant (or similar surfactants) have the potential to kill large numbers of larval amphibians. The interactive effects of predators and the herbicide are best explained by the previously established stratification of the herbicide. It is reasonable to expect that many other pesticides can also stratify in lentic habitats. Perhaps the most striking discovery was that the herbicide was capable of inducing changes in tadpole morphology in a direction and magnitude that appeared to mimic the adaptive morphological changes induced by predators. Future work should investigate the generality of this phenomenon across all amphibians and across similar types of pesticides. Future studies should also determine the underlying mechanisms of herbicide-induced morphology to determine if herbicides and predator cues activate shared endocrinological pathways.

LITERATURE CITED

Altwegg, R., and H.-U. Reyer. 2003. Patterns of natural selection on size at metamorphosis in water frogs. Evolution 57:872–882.

Babbitt, K. J. 2001. Behaviour and growth of southern leopard frog (*Rana sphenocephala*) tadpoles: effects of food and predation risk. Canadian Journal of Zoology 79:809–814.

Barolo, D. 1993. Reregistration eligibility decision for glyphosate. EPA 738-R- 93-014. Reregistration Report. U.S. Environmental Protection Agency, Washington, D.C., USA.

Barry, M. J. 1998. Endosulfan-enhanced crest induction in *Daphnia longicephala*: Evidence for cholinergic innervation of kairomone receptors. Journal of Plankton Research 20:1219–1231.

Barry, M. J. 1999. The effects of a pesticide on inducible phenotypic plasticity in *Daphnia*. Environmental Pollution 104:217–224.

Barry, M. J. 2000. Effects of endosulfan on *Chaoborus*-induced life-history shifts and morphological defenses in *Daphnia pulex*. Journal of Plankton Research 22:1705–1718.

Baylis, A. D. 2000. Why glyphosate is a global herbicide: strengths, weaknesses and prospects. Pesticide Management Science 56:299–308.

Beketov, M. A., and M. Liess. 2005. Acute contamination with esfenvalerate and food limitation: chronic effects on the mayfly, *Cleon dipterum*. Environmental Toxicology and Chemistry 24:1281–1286.

Bernal, M. H., K. R. Solomon, and G. Carrasquilla. 2009. Toxicity of formulated glyphosate (Glyphos) and Cosmo-Flux to larval Colombian frogs 1. Laboratory acute toxicity. Journal of Toxicology and Environmental Health, Part A 72:961–965.

Boutin, C., K. E. Freemark, and C. J. Keddy. 1995. Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environmental Toxicology and Chemistry 14:1465–1475.

Chen, C. Y., K. M. Hathaway, and C. L. Folt. 2004. Multiple stress effects of Vision® herbicide, pH, and food on zooplankton and larval amphibian species from forest wetlands. Environmental Toxicology and Chemistry 23:823–831.

Clements, W. H., and J. R. Rohr. 2009. Community responses to contaminants: using basic ecological principles to predict ecotoxicological effects. Environmental Toxicology and Chemistry 28:1789–1800.

Dinehart, S. K., L. M. Smith, S. T. McMurry, P. N. Smith, T. A. Anderson, and D. A. Haukos. 2010. Acute and chronic toxicity of Roundup Weathermax® and Ignite® 280SL to larval *Spea multiplicata* and *S. bombifrons* from the Southern High Plains, USA. Environmental Pollution 158:2610–2617.

Edginton, A. N., P. M. Sheridan, G. R. Stephenson, D. G. Thompson, and H. J. Boermans. 2004. Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. Environmental Toxicology and Chemistry 23:815–822.

Edwards, W. M., G. B. Triplett, Jr., and R. M. Kramer. 1980. A watershed study of glyphosate transport in runoff. Journal of Environmental Quality 9:661–665.

Giesy, J. P., S. Dobson, and K. R. Solomon. 2000. Ecotoxicological risk assessment for Roundup® herbicide. Reviews of Environmental Contamination and Toxicology 167:35–120.

Glennemeier, K. A., and R. J. Denver. 2002. Small changes in whole-body corticosterone content affect larval *Rana pipiens* fitness components. General and Comparative Endocrinology 127:16–25.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

Hanazato, T. 1991. Pesticides as chemical agents inducing helmet formation in *Daphnia ambigua*. Freshwater Biology 26:419–424.

Hanazato, T. 1999. Anthropogenic chemicals (insecticides) disturb natural organic chemical communication in the plankton community. Environmental Pollution 105:137–142.

Hanazato, T., and H. Hirokawa. 2004. Changes in vulnerability of Daphnia to an insecticide application depending on the population phase. Freshwater Biology 49:402–409.

Howe, C. M., M. Berrill, P. D. Pauli, C. C. Helbring, K. Werry, and N. Veldhoen. 2004. Toxicity of glyphosate-cased pesticides to four North American frog species. Environmental Toxicology and Chemistry 23:1928–1938.

Jones, D. K., J. R. Hammond, and R. A. Relyea. 2010. Roundup® and amphibians: the importance of concentration, application time, and wetland stratification. Environmental Toxicology and Chemistry 29:2016–2025.

Jones, D. K., J. R. Hammond, and R. A. Relyea. 2011. Roundup® and amphibians: Competitive stress can make the herbicide Roundup® more deadly to larval amphibians. Environmental Toxicology and Chemistry 30:446–454.

Lajmanovich, R. C., M. T. Sandoval, and P. M. Peltzer. 2003. Induction of mortality and malformation in *Scinax nasicus* tadpoles exposed to glyphosate formulations. Bulletin of Environmental Contamination and Toxicology 70:612–618.

Lima, S. L. 2002. Putting predators back into behavioral predator-prey interactions. Trends in Ecology and Evolution 17:70–75.

Ma, S., S. C. Kassinos, D. F. Kassinos, and E. Akylas. 2008. Modeling the impact of water withdrawal schemes on the transport of pesticides in the Kouris Dam (Cyprus). Global NEST Journal 10:350–358.

Mann, R. M., and J. R. Bidwell. 1999. The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. Archives of Environmental Contamination and Toxicology 36:193–199.

Miner, B. G., S. E. Sultan, S. G. Morgan, D. K. Padilla, and R. A. Relyea. 2005. Ecological consequences of phenotypic plasticity. Trends in Ecology and Evolution 20:687–692.

Oda, S., Y. Kato, H. Watanabe, N. Tatarazako, and T. Iguchiz. 2011. Morphological changes in *Daphnia galeata* induce by a crustacean terpenoid hormone and its analog. Environmental Toxicology and Chemistry 30:232–238.

Payton, M. E., M. H. Greenstone, and N. Schenker. 2003. Overlapping confidence intervals or standard error intervals: what do they mean in terms of statistical significance? Journal of Insect Science 3:34.

Perez, G. L., et al. 2007. Effects of the herbicide Roundup on freshwater microbial communities: a mesocosm study. Ecological Applications 17:2310–2322.

Postma, J. F., M. C. Buckert-de Jogn, N. Staats, and C. Davids. 1994. Chronic toxicity of cadmium to *Chironomus riparius* (Diptera: Chironomidae) at different food levels. Archives of Environmental Contamination and Toxicology 26:143–148.

Quinn, G. P., and M. J. Keough. 2002. Experimental design and data analysis for biologists. Cambridge University Press, Cambridge, UK.

Relyea, R. A. 2000. Trait-mediated effects in larval anurans: Reversing competition with the threat of predation. Ecology 81:2278–2289.

Relyea, R. A. 2001. Morphological and behavioral plasticity of larval anurans in response to different predators. Ecology 82:523–540.

Relyea, R. A. 2002a. Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. Ecological Monographs 72:523–540.

Relyea, R. A. 2002b. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs 72:77–93.

Relyea, R. A. 2003a. How prey respond to combined predators: a review and an empirical test. Ecology 84:1827–1839.

Relyea, R. A. 2003b. Predator cues and pesticides: a double dose of danger for amphibians. Ecological Applications 13:1515–1521.

Relyea, R. A. 2004a. Fine-tuned phenotypes: tadpole plasticity under 16 combinations of predators and competitors. Ecology 85:172–179.

Relyea, R. A. 2004b. Growth and survival of five amphibian species exposed to combinations of pesticides. Environmental Toxicology and Chemistry 23:1737–1742.

Relyea, R. A. 2004c. Synergistic impacts of malathion and predatory stress on six species of North American tadpoles. Environmental Toxicology and Chemistry 23:1080–1084.

Relyea, R. A. 2005a. The heritability of inducible defenses in tadpoles. Journal of Evolutionary Biology 18:856–866.

Relyea, R. A. 2005b. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. Ecological Applications 15:618–627.

Relyea, R. A. 2005c. The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecological Applications 15:1118–1124.

Relyea, R. A. 2005d. The lethal impacts of Roundup and predatory stress on six species of North American tadpoles. Archives of Environmental Contamination and Toxicology 48:351–357.

Relyea, R. A. 2006. The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities: response. Ecological Applications 15:1527–1532.

Relyea, R. A. 2011. Amphibians are not ready for Roundup®. Pages 267–300 in J. Elliott, C. Bishop, and C. Morrisey, editors. Wildlife ecotoxicology—forensic approaches. Springer, New York, New York, USA.

Relyea, R. A., and J. R. Auld. 2004. Having the guts to compete: How intestinal plasticity explains costs of inducible defenses. Ecology Letters 7:869–875.

Relyea, R. A., and J. R. Auld. 2005. Predator- and competitor-induced plasticity: How changes in foraging morphology affect phenotypic trade-offs. Ecology 86:1723–1729.

Relyea, R. A., and J. T. Hoverman. 2006. Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology Letters 9:1157–1171.

Relyea, R. A., and D. K. Jones. 2009. The toxicity of Roundup Original MAX® to 13 species of larval amphibians. Environmental Toxicology and Chemistry 28:2004–2008.

Relyea, R. A., and N. Mills. 2001. Predator-induced stress makes pesticide carbaryl more deadly to gray treefrog tadpoles (Hyla versicolor). Proceedings of the National Academy of Sciences USA 98:2491–2496.

Relyea, R. A., N. M. Schoeppner, and J. T. Hoverman. 2005. Pesticides and amphibians: the importance of community context. Ecological Applications 15:1125–1134.

Relyea, R. A., and E. E. Werner. 1999. Quantifying the relation between predator-induced behavior and growth performance in larval anurans. Ecology 80:2117–2124.

Sakamoto, M., K.-H. Change, and T. Hanazato. 2006. Inhibition of development of anti-predator morphology in the small cladoceran Bosmina by an insecticide: Impact of an anthropogenic chemical on prey–predator interactions. Freshwater Biology 51:1974–1983.

Schoeppner, N. M., and R. A. Relyea. 2005. Damage, digestion, and defense: The roles of alarm cues and kairomones for inducing prey defenses. Ecology Letters 8:505–512.

Schoeppner, N. M., and R. A. Relyea. 2009. Interpreting the smells of predation: How alarm cues and kairomones induce different prey defenses. Functional Ecology 23:1114–1121.

Semlitsch, R. D., D. C. Scott, and J. H. K. Pechmann. 1988. Time and size at metamorphosis related to adult fitness in Ambystoma talpoideum. Ecology 69:184–192.

Smith, D. C. 1987. Adult recruitment in chorus frogs: effects of size and date at metamorphosis. Ecology 68:344–350.

Solomon, K. R., and D. G. Thompson. 2003. Ecological risk assessment for aquatic organisms from over-water uses of glyphosate. Journal of Toxicology and Environmental Health—Part B—Critical Reviews 6:289–324.

Sudo, M., T. Kawachi, Y. Hida, and T. Kunimatsu. 2004. Spatial distribution and seasonal changes of pesticides in Lake Biwa, Japan. Limnology 5:77–86.

Teplitsky, C., H. Piha, A. Laurila, and J. Merilä. 2005. Common pesticide increases costs of antipredator defenses in Rana temporaria tadpoles. Environmental Science and Technology 39:6079–6085.

Thompson, D. G., B. F. Wojtaszek, B. Staznik, D. T. Chartrand, and G. R. Stephenson. 2004. Chemical and biomonitoring to assess potential acute effects of Vision® herbicide on native amphibian larvae in forest wetlands. Environmental Toxicology and Chemistry 23:843–849.

Tsui, M. T. K., and L. M. Chi. 2003. Aquatic toxicity of glyphosate-based formulations: Comparison between different organisms and the effects of environmental factors. Chemosphere 52:1189–1197.

Van Buskirk, J. 2002. A comparative test of the adaptive plasticity hypothesis: Relationships between habitat and phenotype in anuran larvae. American Naturalist 160:87–102.

Weis, J. S., G. Smith, T. Zhou, C. Santiago-Bass, and P. Weis. 2001. Effects of contaminants on behavior: Biochemical mechanisms and ecological consequences. BioScience 51:209–217.

Wojtaszek, B. F., B. Staznik, D. T. Chartrand, G. R. Stephenson, and D. G. Thompson. 2004. Effects of Vision® herbicide on mortality, avoidance response, and growth of amphibian larvae in two forest wetlands. Environmental Contamination and Toxicology 23:832–842.