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The Lethal Impacts of Roundup and Predatory Stress on Six Species of North American Tadpoles

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Abstract. The decline in amphibians across the globe has sparked a search for the causes, and recent evidence suggests a connection with pesticides. However, for most pesticides, tests on amphibians are rare and conducted only for short durations (1 to 4 days) and without natural stressors. Recent studies have discovered that the stress of predator cues in the water can make insecticides much more lethal to larval amphibians, but it is unknown whether this phenomenon can be generalized to other types of pesticides. Using six species of North American amphibian larvae (*Rana sylvatica*, *R. pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*), I examined the impact of a globally common herbicide (Roundup) on the survival of tadpoles for 16 days with and without the chemical cues emitted by predatory newts (*Notophthalmus viridescens*). $LC50_{16\text{-d}}$ estimates varied from 0.55 to 2.52 mg of active ingredient (AI)/L, which was considerably lower than the few previous studies using Roundup (1.5 to 15.5 mg AI/L). Moreover, in one of the six species tested (*R. sylvatica*), the addition of predatory stress made Roundup twice as lethal. This discovery suggests that synergistic interactions between predatory stress and pesticides may indeed be a generalizable phenomenon in amphibians that occurs with a wide variety of pesticides.

The global decline in amphibians came to the world's attention in the beginning of the last decade and was vigorously debated (Pechmann *et al*. 1991; Blaustein *et al*. 1994; Pechmann and Wilbur 1994; Fisher and Shaffer 1996; Wake 1998). Since that time, surveys of natural populations have become more extensive, and the general consensus is that some species are decreasing, whereas others have remained relatively stable (Fisher and Shaffer 1996; Wake 1998; Alford and Richards 1999; Houlihan *et al*. 2001; Davidson *et al*. 2002; however, see Skelly *et al*. 2003 for assessing the importance of different survey protocols). The apparent causes of these decreases are diverse and include habitat destruction, disease, parasites, invasive species, ultraviolet (UV) radiation, and pesticides (Berger *et al*. 1998; Alford and Richards 1999; LeNoir *et al*. 1999; Kiesecker *et al*. 2001; Relyea and Mills 2001; Sparling *et al*. 2001; Blaustein and Kiesecker 2002; Davidson *et al*. 2002; Hayes *et al*. 2002).

Pesticides are receiving increased attention as a potential cause of amphibian declines. Surveys of natural populations have shown correlations between population declines and proximity to agricultural lands (Davidson *et al*. 2002; Bishop *et al*. 1999; LeNoir *et al*. 1999; Sparling *et al*. 2001). However, assessing the impact of pesticides on amphibian populations is actually quite difficult because for most pesticides, we have few data on concentrations in nature, and we have few studies on pesticide impacts on amphibians. The reason for this paucity of data is that federal regulations for registering pesticides require testing birds, mammals, fish, and aquatic invertebrates but not amphibians (Cooney 1995). As a result, we often estimate the toxicity to amphibians based on other groups (e.g., fish). However, the impact of a pesticide on amphibians can be very different from the impacts on fish or aquatic invertebrates (Relyea 2003, 2004a, 2004b).

Because current regulations do not require testing of amphibians, many globally common pesticides have rarely been tested on amphibians. For example, one of our most frequently used pesticides is glyphosate, a broad-spectrum herbicide that is sold under a wide variety of commercial formulations including Roundup (Monsanto, St. Louis, MO) and Rodeo. Glyphosate is the second most widely used pesticide in the United States. It is currently applied to 8.2 million ha of cropland in the United States including 2 to 3 million kg for home and garden applications and 4 to 6 million kg for commercial and industrial applications (Aspelin and Grube 1999; National Pesticide Use Database www.ncfap.org/database/default.htm). Whereas glyphosate has been widely tested on birds, mammals, invertebrates, and fish, tests on amphibians have been rather limited until recently (Mann and Bidwell 1999; Perkins *et al*. 2000; Smith 2001; Lajmanovich *et al*. 2003; Edginton *et al*. 2004; Thompson *et al*. 2004; Wojtaszek *et al*. 2004). Despite the widespread use of glyphosate in North America, its effect on North American amphibians appears to have been tested in only a few species (Smith 2001; Edginton *et al*. 2004; Thompson *et al*. 2004; Wojtaszek *et al*. 2004). Thus, the need to test the impact of glyphosate on North American amphibians is paramount.

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When we test the impact of pesticides on nontarget organisms such as amphibians, we ultimately wish to know the lethality under natural conditions. However, in standard tests, we typically remove all aspects of the species' natural ecology (U. S. Environmental Protection Agency [USEPA] 1985a, 1985b; Cooney 1995; American Society for Testing and Materials 1996). For example, most tests are conducted for short durations (1 to 4 days) and in the absence of most abiotic and biotic factors that occur in natural habitats. In nature, many species can be exposed to pesticides for longer durations (depending on site conditions) and in combination with numerous factors that can substantially alter the lethality of pesticides: temperature, pH, UV light, competition, and predator-induced stress (Lohner and Fisher 1990; Zaga *et al*. 1998; Boone and Bridges 1999; Boone and Semlitsch 2001, 2002; Relyea and Mills 2001; Relyea 2003, 2004a). For instance, two commonly used insecticides (carbaryl and malathion), which are slightly lethal to larval amphibians at low concentrations, can become highly lethal when combined with the stress of chemical cues produced by predators (*Ambystoma maculatum* or *Notophthalmus viridescens*; Relyea and Mills 2001; Relyea 2003, 2004a). Because living with predators is a common circumstance for most amphibians, amphibians exposed to pesticides will also be exposed to predatory stress. The synergistic interaction between pesticides and predator cues has been tested only with carbaryl and malathion, two broad-spectrum insecticides that inhibit acetylcholine esterase. Thus, the synergy experienced with predator cues may be restricted to only these insecticides (or to insecticides with similar modes of action). However, if synergistic interactions occur with other pesticides that have different modes of action (e.g., herbicides), then many pesticides may become more lethal under natural ecologic conditions.

In this study, I tested the effects of the herbicide Roundup on six species of larval amphibians from North America (wood frogs, *Rana sylvatica*; leopard frogs, *R. pipiens*; green frogs, *R. clamitans*; bullfrogs, *R. catesbeiana*; American toads, *Bufo americanus*; and gray tree frogs, *Hyla versicolor*). These species are not currently decreasing, but they are a diverse set of species that span a large geographic range and can provide insights into the lethality of Roundup. To determine if Roundup becomes more lethal when combined with predatory stress, I exposed each species to Roundup in the presence and absence of predator chemical cues. All collected (and cited) data are reported in units of milligrams active ingredient per liter (mg AI/L).

Roundup Background

Roundup is a broad-spectrum herbicide composed of both the active ingredient (glyphosate) and a surfactant that enables penetration of plant cuticles (polyethoxylated tallowamine [POEA]). The half-lives of glyphosate and POEA are 7 to 70 days and 21 to 28 days, respectively, depending on site conditions (USEPA 1992; Giesy *et al*. 2000). At current application rates, a water body with a mean depth of 15 cm and no intercepting vegetation can have a maximum concentration of 3.7 mg AI/L (Giesy *et al*. 2000). In natural habitats, Roundup has been detected at concentrations of 0.1 to 2.3 mg AI/L (Newton *et al*. 1984; Goldsborough and Brown 1989; Feng *et al*. 1990; Horner 1990). At these concentrations, Roundup is moderately lethal or nonlethal to aquatic invertebrates (LC50 of 3.5 to 323 mg AI/L) and fish (LC50 of 2.8 to 25 mg AI/L). The few studies existing suggest that Roundup is moderately lethal to amphibians ($LC50_{48\text{-h}}$ = 1.5 to 15.5 mg AI/L; Giesy *et al*. 2000; Edginton *et al*. 2004). This moderate toxicity appears to be caused not by the active ingredient (glyphosate) but by the POEA surfactant (Mann and Bidwell 1999; Perkins *et al*. 2000; Tsui and Chu 2003).

Methods

The six species of larval amphibians breed at different times of the year, so I conducted 6 separate experiments. Each experiment used a randomized block design with each block containing a factorial combination of predator cues (present or absent) crossed with 6 concentrations of Roundup (0, 0.1, 1, 5, 10, and 20 mg AI/L). The 12 treatment combinations were replicated 4 times (4 spatial blocks) for a total of 48 experimental units/species. The experimental units were 10-L plastic tubs containing 7.8 L charcoal-filtered, UV-irradiated well water. All experiments were conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology during 2002.

I collected all 6 species of tadpoles as newly oviposited eggs in ponds and marshes from northwestern Pennsylvania (1 to 15 egg masses/species). Eggs were hatched in aged well water and kept predator naïve until used in the experiments. I initiated the experiments with tadpoles soon after hatching (Gosner 1960), i.e., stage 25. Initial mean mass ±1 SE: wood frogs 40 ± 0.3 mg; leopard frogs 25 ± 2.0 mg; toads 19 ± 1.0 mg; tree frogs 22 ± 2.0 mg; green frogs 14 ± 2.0 mg; and bullfrogs 9 ± 1.0 mg). Each tub contained 10 tadpoles, and the tadpoles were fed a daily ration of ground fish flakes (18% of their body mass). This ration was doubled halfway through the experiments to adjust for growth. Laboratory lighting was set at a day-to-night cycle of 14:10 hours. The experiment ran for 16 days to provide longer-than-typical (1 to 4 days) tests. Sixteen days is a large fraction of the larval period for some tadpole species (American toads and gray tree frogs) but a small fraction of the larval period for other species (green frogs and bullfrogs).

For the pesticide manipulations, I used a commercial form of glyphosate (Roundup) with a concentration of glyphosate (25.2% active ingredient, isopropylamine salt) that was confirmed using high-pressure liquid chromatography analyses (Mississippi State Chemical Laboratory). To achieve the desired glyphosate concentrations (20, 10, 5, 1, and 0.1 mg AI/L), I added 624, 312, 156, 31.2, and 3.1 μl of the Roundup solution, respectively. For the control treatments, I added 624 μl water. To prevent the tub water from fouling, I changed the water every 4 days and reapplied the chemical treatments (i.e., static-renewal tests). This protocol has been successfully used in several previous experiments without any problems of impaired water quality (e.g., decreased oxygen or increased ammonia; Relyea and Mills 2001; Relyea 2003, 2004a).

Predator cues were created by using caged predators, which emit chemical cues that diffuse through water and induce prey stress (Kats and Dill 1998; Relyea 2001, 2003). I chose adult red-spotted newts (*Notophthalmus viridescens*) as the predator because newts coexist with all six tadpole species. Newts were caged in 250-ml plastic cups (screened over the open end), and one caged newt was added to each tub assigned the predator treatment. The newts were fed conspecific tadpoles, approximately 100 mg tadpoles every 2 days. No-predator tubs received an empty cage that was lifted every 2 days to equalize disturbance across treatments.

The number of surviving tadpoles was counted each day and any dead tadpoles were removed. I measured the temperature and pH of

Table 1. The results (*p* values) of six separate repeated-measures ANOVAs from analyses of how six species of larval amphibians (in six separate experiments) survived under six concentrations of Roundup crossed with the presence or absence of predator cues with time

Factors (*df*)	Bullfrog	Green frog	Gray treefrog	American toad	Leopard frog	Wood frog
Roundup (5)	<0.001	<0.001	<0.001	<0.001	< 0.001	<0.001
Predator (1)	0.480	0.819	0.566	0.839	0.915	0.072
Roundup * Predator (5)	0.511	0.962	0.945	0.333	0.985	0.059
Time (15)	<0.001	0.001	0.029	<0.001	< 0.001	<0.001
Time * Roundup (75)	0.879	0.535	0.151	<0.001	0.004	<0.001
Time * predator (15)	0.809	1.000	0.974	0.407	0.074	0.001
Time * Roundup * predator (75)	0.993	0.963	0.898	0.503	0.667	<0.001

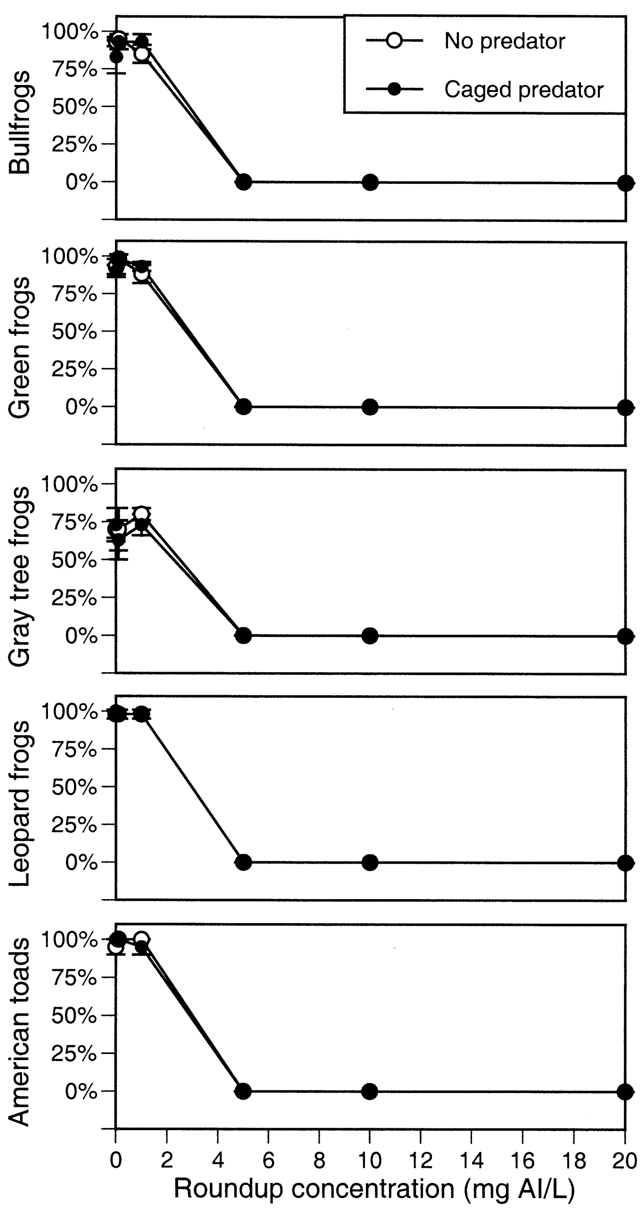

Fig. 1. Survival (mean ± 1 SE) of tadpoles when exposed to a factorial combination of predator cues (absent = open symbols, present = closed symbols) crossed with six concentrations of Roundup. For these five species, there were no Roundup-by-predator interactions.

all tubs midway through the experiment (just before the second water change). The pH ranged from 7.8 to 8.3 among treatments. Temperature varied slightly among species, but the range for each species remained narrow among treatments (wood frogs 18.5 to 18.9°C, leopard frogs 18.6°C to 18.8°C, toads 20.1°C to 20.2°C, tree frogs 20.1°C to 20.3°C, green frogs 21.2°C to 21.3°C, and bullfrogs 21.3°C to 21.4°C). The experiments were terminated after 16 days.

Statistical Analysis

The response variable of interest was the proportion of tadpoles surviving in a tub. The treatments did not have homogeneous errors because survival under high concentrations of Roundup was 0% in all replicates (violating an assumption of parametric analyses). Thus, I conducted nonparametric analyses by first ranking the data and then conducting analyses of variance. The main effects of block and all two- and three-way block interactions with glyphosate and predators were not significant, so their degrees of freedom were pooled with the error term. To estimate LC50 values for each species, I used standard probit regression analyses.

Results

Roundup had significant effects on the survival of all six amphibian species (Table 1). For five of the six species (bullfrogs, green frogs, gray tree frogs, leopard frogs, and American toads), survival was affected by Roundup but not by predator cues or the Roundup-by-predator interaction (Fig. 1). All five species experienced 0% survival with 5 to 20 mg AI/L. At 1.0 and 0.1 mg AI/L, survival improved and was similar to controls ($p > 0.1$). The estimated $LC50_{16\text{-d}}$ values for the five species across both predator treatments ranged from 1.3 to 2.5 mg AI/L (Table 2).

In contrast to the other five species, wood frog survival was affected by Roundup, predator cues, and their interaction (Table 1 and Fig. 2). Survival was 0% in 5 to 20 mg AI/L regardless of predator treatment. However, at 1.0 mg AI/L, survival was 65% with predator cues absent but only 30% with predator cues present ($p = 0.035$). There also was a trend of lower survival with predators at 0.1 mg AI/L, but this trend was not significant ($p = 0.304$). At 0.1 mg AI/L, survival across predator treatments improved to 73%, which was still significantly lower than the 83% survival of controls ($p = 0.010$). The estimated LC50 for wood frogs was 1.32 mg/L without predator cues and 0.55 mg AI/L with predator cues (Table 2).

Table 2. LC50 values for larval amphibians reared with different concentrations of Roundup

Species	$LC50_{16\text{–}d}$
Bull frog	2.07
Green frog	2.17
Gray tree frogs	1.35
Leopard frogs	2.46
American toads	2.52
Wood frogs (no predator)	1.32
Wood frogs (caged predator)	0.55

Discussion

The experiments indicated that the LC50 estimates for the six amphibian species ranged from 0.55 to 2.52 mg AI/L. Under current classifications, this means that Roundup is moderately toxic (1 to 10 mg AI/L) to highly toxic (0.1 to 1 mg AI/L) to these amphibians (Giesy *et al.* 2000). Previous work has concluded that Roundup is only slightly to moderately toxic to larval amphibians, but this work is based on relatively few species. In four species of Australian tadpoles (*Crinia insignifera*, *Heleioporus eyrei*, *Limnodynastes dorsalis*, and *Litoria moorei*), Mann and Bidwell (1999) estimated that the $LC50_{48\text{-h}}$ values ranged from 3.9 to 15.5 mg AI/L for Roundup, 108 to 161 mg AI/L for technical-grade glyphosate acid, and >450 mg AI/L for glyphosate isopropylamine salt (the latter two formulations lack the POEA surfactant). In *Xenopus laevis* tadpoles, $LC50_{96\text{-h}}$ values were 12.4 mg AI/L for Roundup, 6.8 mg/L for the POEA surfactant, and 9729 mg AI/L for Rodeo (a formulation that lacks any the POEA surfactant; Perkins *et al.* 2000). Another formulation of glyphosate (GLYFOS, a combination of glyphosate and the POEA surfactant) has been tested on South American tadpoles (*Scinax nasicus*) and found to have an $LC50_{48\text{-h}}$ of 1.74 mg AI/L (Lajmanovich *et al.* 2003). In experiments using Vision (a formulation that also includes the POEA surfactant) and three species of tadpoles from Canada (*Bufo americanus, Rana pipiens*, and *R. clamitans*), Edginton *et al.* (2004) found $LC50_{96\text{-h}}$ values of 1.5 to 4.7 mg AI/L. Thus, the LC50 values generated by the current study are relatively low compared with those reported in previous studies. These lower LC50 values are due, at least in part, to the longer duration of the current study. In addition, the previously mentioned studies suggest that the POEA surfactant, rather than the active ingredient (glyphosate), is the likely cause of the high toxicity of Roundup.

Although there are few data on the effects of Roundup on amphibians, there are numerous data on the impact of Roundup on other organisms. Giesy *et al.* (2000) recently completed an extensive review of the glyphosate literature. They found that Roundup is practically nontoxic to birds (based on 3 species) and mammals (based on 5 species). The toxicity of Roundup is variable for freshwater fish (based on 11 species), ranging from moderately toxic in bluegill sunfish (*Lepomis macrochirus*; $LC50_{96\text{-h}}$ of 2.8 mg AI/L) to slightly toxic in rainbow trout (*Oncorhynchus mykiss*; $LC50_{96\text{-h}}$ of 25 mg AI/L). For aquatic invertebrates (based on 8 species), Roundup ranges from moderately toxic in crayfish (*Orconectes nais*; $LC50_{96\text{-h}}$ of 3.5 mg AI/L) to practically nontoxic in mosquito larvae (*Anopheles quadrimaculatus*; $LC50_{24\text{-h}}$ of 323 mg AI/L; Giesy *et al.* 2000). If we estimate the toxicity to amphibians using these data for fish and aquatic invertebrates, we would expect glyphosate to be slightly to moderately toxic to amphibians. However, the current study found that glyphosate is moderately to highly toxic to amphibians.

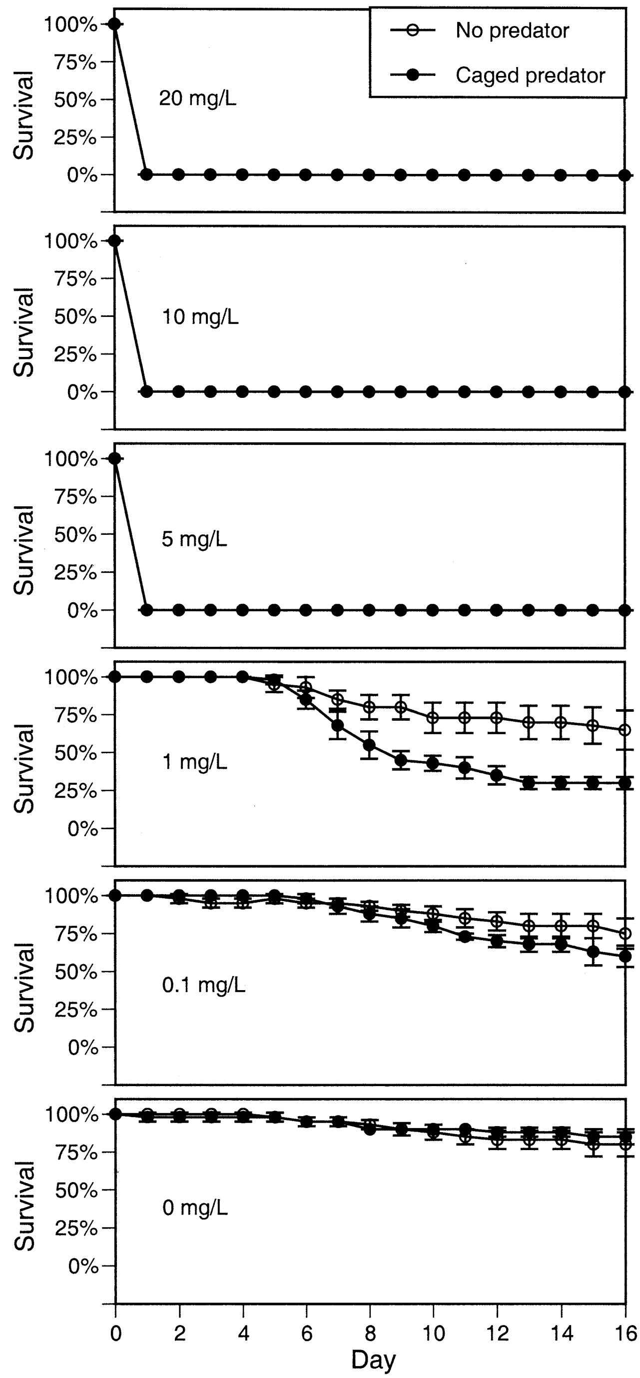

Fig. 2. Survival (mean ± 1 SE) of wood frog tadpoles when exposed to a factorial combination of predator cues (absent = open symbols, present = closed symbols) crossed with six concentrations of Roundup. For this species, there was a significant Roundup-by-predator interaction.

By comparing the LC50 values of the six species with the maximum concentrations in wetlands, we can assess the potential impact of Roundup on amphibians in nature. For a water body 15 cm deep with no overhead vegetation (a condition in which many amphibians live), the maximum concentration of Roundup is estimated to be 3.7 mg AI/L (Giesy *et al.* 2000), and observed concentrations range from 0.1 to 2.3 mg AI/L (Newton *et al*. 1984; Goldsborough and Brown 1989; Feng *et al*. 1990; Horner 1990). Based on the probit regression analyses in this study, 3.7 mg AI/L would kill 90% to 100% of tadpoles from all six species. At 2.3 mg AI/L, Roundup has the potential to kill 40% to 98% of the individuals of all six species. Thus, at the high end of observed concentrations, Roundup has the potential to cause widespread death in amphibians. However, recent experiments using field enclosures and the glyphosate formulation Vision, researchers found no significant impact of the herbicide on larval leopard frogs and green frogs in Canada (Thompson *et al*. 2004; Wojtaszek *et al*. 2004). Given the highly variable results of glyphosate formulations on larval amphibians, it is clear that much more work needs to be conducted to arrive at a general consensus of the impacts on amphibians. Moreover, whether deaths from a pesticide actually contribute to amphibian declines depends critically on whether pesticide-related death is additive or compensatory with other natural causes of death (e.g., predation, disease).

In one of the six species (wood frogs), Roundup became twice as toxic when combined with predator-induced stress. Although the mechanism underlying this synergy is unknown, previous work has shown that the synergy is not caused by caged predators altering the concentrations of ammonia or dissolved oxygen in the water (Relyea and Mills 2001; Relyea 2003, 2004a). This synergy between pesticides and predator cues was first discovered for the insecticide carbaryl (a carbamate that inhibits acetylcholine esterase, commercial name Sevin). Carbaryl became more deadly to tadpoles of gray tree frogs, bullfrogs, and green frogs when combined with the chemical cues emitted by salamander predators (*Notophthalmus viridescens* and *Ambystoma maculatum* [Relyea and Mills 2001; Relyea 2003]). A subsequent series of experiments with malathion (an organophosphate that also inhibits acetylcholine esterase) also found synergistic interactions, but only for gray tree frogs (Relyea 2004a). The discovery of synergistic interactions between predator cues and Roundup indicates that the phenomenon is not restricted to carbaryl and malathion (and possibly other carbamates and organophosphates). Rather, it suggests that predatory stress may make a variety of pesticides more deadly to amphibians. This is important because most amphibians live with the stress of predators. Although the study of synergistic interactions between pesticides and predator stress has currently only focused on amphibians, there is no inherent reason to believe that the synergies cannot happen in other taxonomic groups. Moreover, synergistic interactions with Roundup are not restricted to predatory stress. For example, Tsui and Chu (2003) recently demonstrated that zooplankton (*Ceriodaphnia dubia*) experience synergistic interactions between Roundup and suspended soil particles and between Roundup and pH. In short, numerous potential synergistic interactions exist between pesticides and natural ecologic environments.

The results also underscore the importance of running toxicity experiments for longer than the typical 1- to 4-day duration. For some species, extending the duration of exposure had minimal effects on survival. However, for other species, extending the exposure time had a profound negative effect. For example, wood frog survival was excellent (100%) during the first 4 days with 1 mg AI/L. By day 6, however, survival began to decrease; after 16 days survival was decreased to 65% without predator cues and to only 30% with predator cues. Although we expect higher death rates with increased exposure duration (Cooney 1995), it is not clear why some species' death rates increase only marginally while other species' death rates increase substantially. Exposure times to Roundup may be short under high pH conditions, but Roundup breaks down much more slowly under low pH conditions, thus causing longer exposure times (USEPA 1992; Giesy *et al.* 2000). In short, by running experiments for longer durations, we can observe substantially higher estimates of a pesticide's toxicity (Relyea and Mills 2001; Relyea 2003, 2004a).

Conclusion

The global decline of amphibians is of paramount importance to both scientists and the general public (Pechmann *et al*. 1991; Blaustein *et al*. 1994; Pechmann and Wilbur 1994; Wake 1998; Houlihan *et al*. 2001). Like most biological problems, the causes are multifaceted and complex (Alford and Richards 1999; Kiesecker *et al*. 2001; Berger *et al*. 1998; Blaustein and Kiesecker 2002). Recent studies have implicated pesticides as important players in amphibian declines (Bishop *et al*. 1999; LeNoir *et al.* 1999; Sparling *et al*. 2001; Relyea and Mills 2001; Davidson *et al*. 2002; Hayes *et al*. 2002), yet we have suffered from a lack of data on how most globally common pesticides affect amphibian survival. The current study demonstrates that when we use realistic exposure times and the frequently occurring stress of predators found in natural ecologic communities, one of our most widely applied herbicides (Roundup) has the potential to kill many species of amphibians. Given that 6 to 9 million kg glyphosate are currently used annually across >8 million ha in the United States (Aspelin and Grube 1999; National Pesticide Use Database, www.ncfap.org/database/default.htm), Roundup with the POEA surfactant has the potential to play a major role in amphibian declines. However, it is worthy to note that the manufacturer of Roundup (Monsanto Corp.) has recently released an additional formulation of glyphosate (Roundup Biactive), which contains a different (but unspecified) surfactant that is reported to be less toxic (Tsui and Chu 2003). The impact of this new formulation is certainly worthy of testing on larval amphibians.

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