PREDATOR CUES AND PESTICIDES: A DOUBLE DOSE OF DANGER FOR AMPHIBIANS

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Abstract.   Amphibians are declining globally, and biologists have struggled to identify the causes. Pesticides may play a role in these declines, but pesticide concentrations in nature often are low and considered sublethal. Past research has found that the globally common pesticide carbaryl can become more lethal under different environmental conditions including differences in temperature and competition. A recent study has found that predatory stress, a situation common for most amphibians, can make carbaryl 2–4 times more deadly to gray tree frogs (*Hyla versicolor*). To determine whether this is a general phenomenon in amphibians, I examined how carbaryl affected the survival of six amphibian species in the presence and absence of predatory stress. Higher concentrations of carbaryl caused higher mortality. In two of the six species, carbaryl became even more lethal when combined with predatory stress (up to 46 times more lethal). This suggests that apparently safe concentrations of carbaryl (and perhaps other pesticides with similar modes of action) can become more deadly to some amphibian species when combined with predator cues.

Key words: amphibian decline; Bufo americanus; Hyla versicolor; *pesticide concentrations; phenotypic plasticity; predation;* Rana catesbeiana; Rana clamitans; Rana pipiens; Rana sylvatica; *synergy.*

INTRODUCTION

The decline of amphibian populations over much of the globe began to attract attention a decade ago (Blaustein and Wake 1990, Blaustein 1994, Blaustein et al. 1994, Pechmann and Wilbur 1994) and is now a well-documented phenomenon (Wake 1998, Alford and Richards 1999, Davidson et al. 2001, Houlihan et al. 2001). While habitat loss is undeniably the major cause of amphibian declines, several additional causes have been proposed including UV radiation, predators, parasites, disease, and pesticides (Berger et al. 1998, Alford and Richards 1999, Davidson et al. 2001, Kiesecker et al. 2001). The role of pesticides in amphibian declines has been especially unclear. While nearly 1×10^9 kg of pesticides are used globally (USEPA 1997), only recently have researchers identified correlations between declining amphibian populations and either the proximity to larger amounts of agricultural lands (Davidson et al. 2001) or decreased acetylcholine esterase activity (a signal of carbamate and organophosphate presence; Sparling et al. 2001). However, in these correlative studies, the concentrations of pesticides detected in declining populations have been below what is thought to be lethal to amphibians based on traditional toxicology studies (LeNoir et al. 1999, Sparling et al. 2001).

A growing number of studies suggest that the lethality of pesticides can be highly dependent on the experimental context. For example, the insecticide carbaryl (1-naphthyl N-methylcarbamate, commercial name: Sevin; Rhone-Poulenc, Research Triangle Park, North Carolina, USA) can become more lethal to aquatic animals under warmer temperatures (Lohner and Fisher 1990, Boone and Bridges 1999; but see Bridges and Boone [2003]) and more or less lethal when combined with competitive interactions from other species (Boone and Semlitsch 2001, 2002). A recent study found that carbaryl also becomes more lethal to gray tree frog tadpoles (*Hyla versicolor*) when combined with the chemical cues emitted by predators (Relyea and Mills 2001). This discovery was important because most habitats contain predators, so most amphibians that experience pesticides will simultaneously experience predatory stress (Kats and Dill 1998, Relyea and Werner 1999, Relyea 2001). However, the combination of carbaryl and predatory stress has only been examined in one species. In this study, I took a more broadly comparative approach and tested for the synergistic effects between carbaryl and predatory stress using six species of amphibians from three taxonomic families (wood frogs, *Rana sylvatica*; leopard frogs, *R. pipiens*; green frogs, *R. clamitans*; bullfrogs, *R. catesbeiana*; American toads, *Bufo americanus*; and gray tree frogs). While these species are not experiencing declines in the eastern United States, they do span a wide geographic and taxonomic range and therefore can serve to assess the general lethality of carbaryl and the prevalence of synergistic interactions with predatory stress.

METHODS

To examine the interactive effects of predator cues and carbaryl, I reared six species of tadpoles in separate

PLATE. 1. An adult newt predator hunting for tadpoles and causing predatory stress on tadpoles (photo by Jason Hoverman).

experiments (due to differences in breeding phenology) at the University of Pittsburgh's Pymatuning Laboratory of Ecology, Pittsburgh, Pennsylvania, USA. I used a randomized block design with a factorial combination of two predator cue treatments (the presence or absence of chemical cues; Petranka et al. 1987, Kats et al. 1988) and six concentrations of carbaryl (6.5, 3.2, 1.6, 0.3, 0.03, and 0.0 mg/L). Carbaryl concentrations in aquatic habitats have been documented at levels up to 4.8 mg/L (Norris et al. 1983, Peterson et al. 1994).

The 12 treatment combinations were replicated four times for a total of 48 experimental units (10-L plastic tubs containing 7.8 L of filtered well water) in each of the six experiments. Carbaryl was added to the tubs using a commercial form of carbaryl (Sevin) whose stock concentration (73 g/L) was determined using high-pressure liquid chromatography analyses (Mississippi State Chemical Laboratory, Mississippi State, Mississippi, USA). Previous work has demonstrated no difference between commercial forms of carbaryl and technical grade carbaryl on amphibian growth and survival (C. M. Bridges, *personal communication*). For the 6.5, 3.2, 1.6, 0.3, and 0.03 mg/L carbaryl treat-

ments, I pipetted 693, 347, 173, 35, and 3.5 μL of Sevin into the tubs, respectively. For 0 mg/L, I added 693 μL of water.

Tadpoles were collected as newly oviposited eggs (5–10 egg masses per species) from several ponds and wetlands surrounding Pymatuning Lake in northwest Pennsylvania. I hatched the eggs in aged well water to keep the animals predator-naive. Newly hatched tadpoles (Gosner stage 25; Gosner 1960) were used for all experiments (wood frogs, 16.9 ± 1.0 mg; leopard frogs, 19.0 ± 2.0 mg; toads, 19.9 ± 1.3 mg; gray tree frogs, 20.9 ± 1.7 mg; green frogs, 8.7 ± 0.4 mg; bullfrogs, 12.1 ± 1.0 mg; means $\pm$ 1 SE). I placed 10 tadpoles into each of the water tubs and conducted the experiments for 16 d (i.e., a chronic test). I fed the tadpoles every 2 d using ground fish flakes at a ration of 18% of initial mass per day. After 8 d, I doubled this ration because the tadpoles had approximately doubled in mass.

Each tub was also equipped with a predator cage constructed of a screened 250 mL plastic cup. In tubs assigned the predator cue treatment, cages contained an adult red-spotted newt *Notophthalmus viridescens* (see Plate 1), a predator that coexists with all of the tadpole species and readily consumes each of them (Collins and Wilbur 1979, Relyea 2001). The predators were fed ~100 mg of conspecific tadpoles in the cup every 2 d to generate the predator cues (Kats et al. 1988). To prevent water fouling, tub water was changed every 4 d and the carbaryl treatments were reapplied (i.e., static renewal tests). Each day I quantified the number of surviving tadpoles and removed any dead tadpoles. The laboratory day : night cycle was held at 14:10 h, water temperature ranged from 18.2° to 20.0°C, and pH ranged from 7.8 to 8.0.

Using the proportion of tadpoles surviving in each tub as my response variable, I analyzed the daily survival data using repeated measures analyses of variance for each species. Due to heteroscedastic errors, I analyzed the data nonparametrically by first ranking the data. The two- and three-way block interactions with carbaryl and predator cues were never significant and, thus, were pooled with the error term. To estimate the

TABLE 1. ANOVA results (*P* values) from the analyses of how six species of larval amphibians (in six separate experiments) survived under six concentrations of the pesticide carbaryl crossed with the presence or absence of predator cues over time in studies conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology.

| Species	Wood frogs	Leopard frogs	American toads	Gray tree frogs	Green frogs	Bullfrogs
Block	0.040	0.263	0.202	0.900	0.487	0.325
Carbaryl	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Predator	<0.001	0.812	0.009	0.748	<0.001	<0.001
Predator × carbaryl	0.464	0.146	0.938	0.564	<0.001	0.005
Time	1.000	1.000	1.000	1.000	1.000	1.000
Time × block	0.090	0.688	0.091	0.194	0.943	0.196
Time × carbaryl	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Time × predator	0.224	0.706	0.036	0.171	0.157	<0.001
Time × carbaryl × predator	<0.001	<0.001	0.204	0.826	<0.001	<0.001
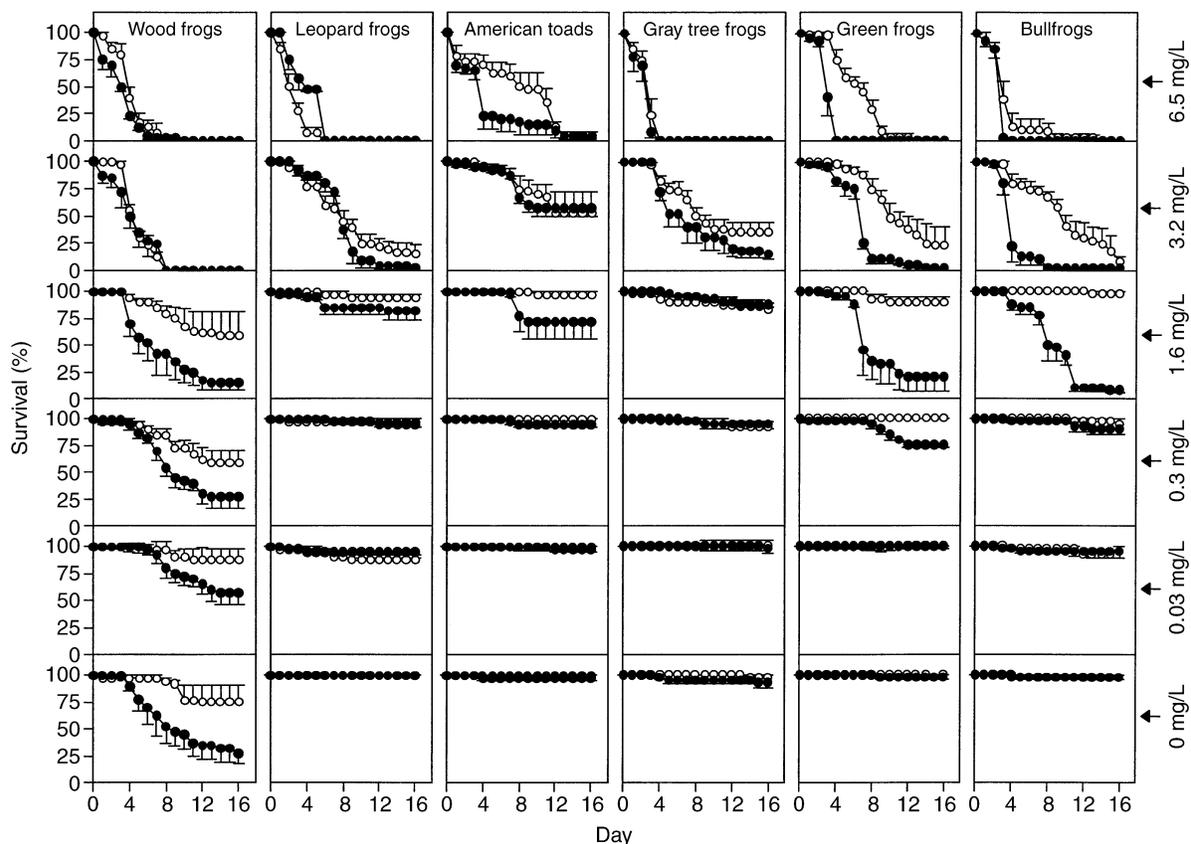
FIG. 1. The survival of six tadpole species after being exposed to a factorial combination of predator cues (absent, open symbols; present, closed symbols) crossed with four concentrations of the pesticide carbaryl. For space consideration, the lowest concentrations of carbaryl are not shown (0.03 mg/L) because this concentrations had little effect on the survival in any of the species. Data are means ± 1 SE.

LC50 values (i.e., the lethal concentration of a pesticide that is expected to kill 50% of a test population) for each species, I used standard probit regression analyses. To determine whether the addition of the predators affected the abiotic conditions in the tubs, I quantified ammonia and oxygen concentrations in three of the six experiments (wood frogs, leopard frogs, and toads) midway through the experiment (on day 8, before the water was changed).

RESULTS

The highest concentrations of carbaryl caused high mortality in all six species (Table 1, Fig. 1). In wood frogs, there were significant effects of carbaryl and predator cues as well as significant time-by-carbaryl and time-by-carbaryl-by-predator interactions. At the highest concentrations (3.2 and 6.5 mg/L), carbaryl killed all of the wood frog tadpoles, regardless of predator cue treatment. However, at lower concentrations, carbaryl became less deadly and similar to the control treatment ($P > 0.1$). Predator cues caused increased mortality in wood frogs, both with low concentrations of carbaryl (0.03–1.6 mg/L) and with no carbaryl present. The LC50 value without predators was 1.2 mg/

L. Mortality was always >50% with predator cues present; thus the LC50 value with predator cues was not estimated.

In leopard frogs, there were significant effects of carbaryl and a significant time-by-carbaryl interaction (Table 1, Fig. 1). The highest concentrations of carbaryl (6.5–3.2 mg/L) killed 100% and 90% of the animals, respectively. At 1.6 mg/L survival improved to 89%, but this was still lower than the control ($P = 0.001$). At lower concentrations of carbaryl (0.3–0.03 mg/L), leopard frog mortality was low and not different from the control ($P > 0.08$). There also was a time-by-carbaryl-by-predator interaction; predator cues improved survival at 6.5 mg/L of carbaryl midway through the experiment, but they had no effect at the other concentrations. This favorable synergy at 6.5 mg/L rapidly declined over time as all of the animals eventually died. The LC50 value across predator treatments was 2.2 mg/L.

In toads, there were significant effects of carbaryl and predators as well as a time-by-carbaryl and a time-by-predator interaction (Table 1, Fig. 1). At the highest carbaryl concentration (6.5 mg/L), 95% of the toads died after 16 d. Midway through the experiment at this concentration, however, it was clear that predator cues

were making carbaryl more deadly, but this synergistic interaction disappeared by the end of the experiment because carbaryl alone eventually killed most of the tadpoles. At the next lower concentration (3.2 mg/L), carbaryl was less deadly, killing only 45% of the tadpoles, but significantly more than the controls ($P <$ 0.001). Mortality continued to decline with less carbaryl (1.6–0.03 mg/L) and was not different from the controls ($P > 0.07$). The LC50 value across predator treatments was 3.4 mg/L.

In gray tree frogs, there was a significant effect of carbaryl and a time-by-carbaryl interaction (Table 1, Fig. 1). Under the highest carbaryl concentration (6.5 mg/L), all of the tree frogs died. At 3.2 mg/L, survival improved to 25%, but was significantly lower than the controls ($P < 0.001$). At 1.6 mg/L, survival improved to 85% but was still lower than the 95% survival in the controls ($P = 0.007$). Under the lowest concentrations (0.3–0.03 mg/L), tadpole survival was similar to the controls ($P > 0.2$). The LC50 value across predator treatments was 2.5 mg/L.

In green frogs, there were significant effects of carbaryl and predator cues as well as predator-by-carbaryl, time-by-carbaryl, time-by-predator, and time-by-predator-by-carbaryl interactions (Table 1, Fig. 1). The widespread interactions occurred because predator cues caused carbaryl to become more deadly to green frogs at 6.5–0.3 mg/L. Under the highest concentration (6.5 mg/L), the synergy was present midway through the experiment but disappeared by the end of the experiment because carbaryl alone eventually killed all of the green frogs. At 3.2–0.3 mg/L, the synergy persisted to the end of the experiment. In the most extreme case (1.6 mg/L), carbaryl killed 10% of the green frogs when predator cues were absent but it killed 80% of the green frogs when predator cues were present (eight times more lethal; $P = 0.011$). Under the lowest carbaryl concentration (0.03 mg/L), there was no effect of carbaryl or predators, causing survival rates that were similar to the controls ($P = 0.457$). The LC50 value was 2.6 mg/L without predator cues and 1.1 mg/L with predator cues.

In bullfrogs, there were significant effects of carbaryl and predator cues as well as significant predator-by-carbaryl, time-by-carbaryl, time-by-predator, and time-by-predator-by-carbaryl interactions (Table 1, Fig. 1). As in the green frogs, the interactions occurred because the higher carbaryl concentrations became more deadly when combined with predator cues. At the highest concentration (6.5 mg/L), a synergistic effect appeared early in the experiment but rapidly disappeared as carbaryl alone eventually killed all of the tadpoles. At intermediate concentrations (3.2 and 1.6 mg/L), the synergistic effect persisted until the end of the experiment. In the most extreme synergy (at 1.6 mg/L), 2% of the bullfrogs died with carbaryl alone but 92% of the tadpoles died when carbaryl was combined with predator cues (46 times more deadly; $P = 0.001$). At the lowest

concentrations (0.3–0.03 mg/L), bullfrog survival was very high and not different from the controls ($P > 0.2$). The LC50 value was 2.3 mg/L without predator cues and 1.0 mg/L with predator cues.

In three of the experiments, I measured ammonia and dissolved oxygen concentrations to determine if the addition of caged predators caused an increase in ammonia (due to metabolic waste products) or a decrease in oxygen due to respiration. For wood frogs, there was no effect of predator cues on dissolved oxygen or ammonia ($P > 0.2$). For leopard frogs, predator cues had no effect on ammonia ($P = 0.121$), but caused a 7% reduction in dissolved oxygen from 5.7 to 5.3 mg/L oxygen ($P < 0.001$). In toads, predator cues were associated with a 14% reduction in ammonia (from 2.2 to 1.9 mg/L ammonia, $P = 0.021$) and a 15% reduction in dissolved oxygen (from 3.4 to 2.9 mg/L oxygen; $P = 0.03$).

DISCUSSION

Higher concentrations of carbaryl caused higher mortality in all six species of tadpoles and the LC50 values in the current study tended to be lower than those previously reported for larval anurans. In this study, LC50 values ranged from 1.0 to 3.4 mg/L. Previous studies, using short exposure durations (3–4 d), have estimated carbaryl LC50 values at 2.5–18 mg/L (Marchal-Segault 1976, Marian et al. 1983, Bridges 1997, Zaga et al. 1998). Given the longer exposure durations in the current study, the lower LC50 values were expected. The exposure durations to carbaryl in nature are highly dependent on the environmental conditions. For example, natural wetlands normally have a range of pH conditions of 5–9; the half-life of carbaryl due to hydrolytic breakdown ranges from 0.1 d at pH = 9 to nearly 4 yr at pH = 6 (Aly and El-Dib 1971, Wauchope and Haque 1973, Sikka et al. 1975, Sharom et al. 1980). Photolytic breakdown can cause 4–7 d half-lives, depending on the amount of shade and the turbidity of the water (Wolfe et al. 1978). Thus, in ponds with low pH and shaded conditions, carbaryl has the potential to remain in the system for at least the duration of the experiments (16 d).

In several species there were synergistic interactions between carbaryl and predator cues (Table 1, Fig. 1). In two species (wood frogs and gray tree frogs), there were no indications of any synergies. Surprisingly, wood frogs experienced higher death with predator cues even when there was no carbaryl present. In two other species (leopard frogs and toads) there were synergistic interactions early in the experiments at high carbaryl concentrations, but the synergies disappeared by the end of the experiments because carbaryl alone eventually killed nearly all of the tadpoles. In the remaining two species (green frogs and bullfrogs), they were large synergistic interactions that persisted throughout the experiments. The predator cues made

carbaryl up to eight times more deadly in green frogs and up to 46 times more deadly in bullfrogs.

In the initial work that discovered synergistic interactions between carbaryl and predator cues, predator cues from spotted salamanders (*Ambystoma maculatum*) made low carbaryl concentrations (0.05–0.54 mg/L) 2–4 times more deadly to gray tree frogs collected in Missouri, USA (Relyea and Mills 2001). Thus, of the six species now tested, persistent synergistic interactions have been found in three species (green frogs, bullfrogs, and gray tree frogs). It is noteworthy that while synergies were found in gray tree frogs from two Missouri populations in the earlier study (using cues from spotted salamander predators), there were no synergies found in gray tree frogs from the Pennsylvania population in the current study (using cues from red-spotted newt predators). This suggests that either different predators cause different synergistic effects or that there are differences between tree frog populations in Pennsylvania and Missouri that result in different susceptibility to the combined effects of carbaryl and predator cues. Both possibilities should be excellent avenues of future research.

The synergistic effects between carbaryl and predator cues represent a multiple stressor phenomenon, but the mechanism is unknown. The synergy might be caused by compounded physiological stress resulting from both predators and the pesticide's inhibition of acetylcholine esterase. Alternatively, the synergy might be caused by changes in abiotic factors caused by the addition of the caged predators. In the three experiments in which I quantified oxygen and ammonia concentrations, I found no increase in ammonia with the addition of predators and a 0–15% reduction in oxygen. This result is consistent with past experiments (Relyea and Mills 2001) in which predators were associated with no increase in ammonia and a 0–9% decrease in oxygen. While it is possible that the predator–pesticide synergy was caused by a reduction in oxygen, the concentrations of oxygen (2.9–5.3 mg/L) are still high relative to the needs of most aquatic organisms, and the synergistic interaction between predator cues and carbaryl exists even when there has been no reduction in oxygen (Relyea and Mills 2001). Thus, the synergy appears not to be driven by any abiotic changes that are associated with the predator's presence. Although the mechanisms are rarely well understood, synergistic interactions between pesticides and other factors have frequently been observed under laboratory conditions including carbaryl and temperature or pH (Lohner and Fisher 1990, Boone and Bridges 1999), carbaryl and UV-B (Zaga et al. 1998), and malathion and the bacterium *Aeromonas hydrophila* (Taylor et al. 1999).

Synergistic survival effects involving carbaryl and biotic factors also have been tested in outdoor mesocosm studies. In one study, Boone and Semlitsch (2001) found that Woodhouse's toads (*Bufo woodhousii*) experienced nearly 0% survival without carbaryl but 30% survival with very high concentrations of carbaryl (7 mg/L), and this survival with carbaryl was greater with high competition than low competition. No such interactions were found for plains and southern leopard frogs (*R. blairi* and *R. sphenocephala*). In a subsequent study, Boone and Semlitsch (2002) found the opposite result; Woodhouse's toad survival declined with very high concentrations of carbaryl (7 mg/L), and this survival was greater with lower competition than high competition. Larval gray tree frogs also suffered lower survival with high concentrations of carbaryl, but this survival was greater at high competition. Larval green frogs showed no interactions. It is unclear why there were contradictory data in these two studies, although it may be the result of different indirect effects operating in the outdoor mesocosms that were used.

A critical question in this type of research is how results observed in the laboratory might reflect pesticide risk under natural conditions. While carbaryl is used around the world, there is no evidence to date that carbaryl in particular has caused amphibian declines nor that killing some fraction of larval amphibians will necessarily decrease population sizes (i.e., compensatory vs. additive mortality). Thus, we are left simply assessing the potential for carbaryl to kill amphibians in nature. Under natural conditions, carbaryl concentrations have been observed as high as 4.8 mg/L. Because laboratory studies indicate that LC50 values can be as low as 1 mg/L, this implies that carbaryl has the potential to cause high rates of amphibian mortality, particularly when combined with predatory stress. Further, even sublethal concentrations of carbaryl can affect tadpole behavior and make tadpoles more susceptible to predation (Bridges 1997, 1999*a, b*). Because the current study used chronic exposure durations of 16 d, the results may be more appropriate for natural wetlands with low pH where the breakdown of carbaryl is much slower; the half-life of carbaryl is 0.1 d at pH $= 9$, 1 d at pH $= 8$, 10 d at pH $= 7$, and 1500 d at pH $= 5–6.5$ (Aly and El-Dib 1971, Sharom et al. 1980). A critical next step is to determine whether synergistic interactions between carbaryl and predators actually occur under more natural conditions.

The impact of carbaryl on amphibian populations is of particular concern because it is a globally common pesticide and one of the most commonly used pesticides in the United States. It is applied to rangelands, forests, oceans, and >100 species of crops as well as 28×10^6 homes and 31×10^6 gardens in the United States (USDA 1989, USEPA 1992). Because carbaryl shares a common mode of action with many other carbamate and organophosphate pesticides (inhibiting acetylcholine esterase), it may serve as a model for other similar pesticides. If so, the synergistic interactions between carbaryl and predator stress may extend to a larger suite of pesticides. Further, because predators are present in the vast majority of aquatic habitats (R. A. Relyea, E. E. Werner, D. K. Skelly, and K. L. Yu-

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rewicz, *unpublished data*), the potential for interactions between pesticides and predatory stress is high. Further investigations need to be conducted with other pesticides to assess the extent of the phenomenon.

Two lines of evidence suggest that pesticides may be playing a role in amphibian declines in the western United States. First, Davidson et al. (2001, 2002) have found associations between declining amphibian populations and the amount of upwind agricultural lands (i.e., potential sources of pesticides). Second, in habitats with declining populations of red-legged frogs (*R. aurora*), yellow-legged frogs (*R. boylii* and *R. muscosa*), and Yosemite toads (*Bufo boreas*), Sparling et al. (2001) have found that Pacific tree frogs (*Hyla regilla*) possess lower acetylcholine esterase activity (a signal of possible carbamate and organophosphate presence) and greater concentrations of organophosphate residues than in habitats in which the populations are not declining. However, while substantial fractions of aerially applied pesticides can enter the atmosphere and be transported downwind (up to 30%; Miller et al. 1996), the concentrations of pesticides detected in aquatic habitats have not been in the lethal range based on traditional toxicology studies (McConnell et al. 1998, LeNoir et al. 1999, Sparling et al. 2001).

The current study suggests that the lethal concentrations of carbaryl (and perhaps other pesticides) can be much lower than we currently appreciate because traditional toxicology studies frequently isolate animals from their natural ecology (including predator cues). When we include some of the natural ecology, even low concentrations of a pesticide can be highly lethal to amphibians. In short, ignoring the relevant ecology can cause incorrect estimates of a pesticide's lethality in nature, yet it is the lethality of pesticides under natural conditions that is of utmost interest. The accumulating evidence strongly suggests that pesticides in nature could be playing a role in the decline of amphibians.

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