

## SYNERGISTIC IMPACTS OF MALATHION AND PREDATORY STRESS ON SIX SPECIES OF NORTH AMERICAN TADPOLES

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**Abstract**—The decline of many amphibian populations is associated with pesticides, but for most pesticides we know little about their toxicity to amphibians. Malathion is a classic example; it is sprayed over aquatic habitats to control mosquitoes that carry malaria and the West Nile virus, yet we know little about its effect on amphibians. I examined the survival of six species of tadpoles (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*) for 16 d in the presence or absence of predatory stress and six concentrations of malathion. Malathion was moderately toxic to all species of tadpoles (median lethal concentration [LC50] values, the concentration estimated to kill 50% of a test population, ranged from 1.25–5.9 mg/L). These values are within the range of values reported for the few amphibians that have been tested (0.2–42 mg/L). In one of the six species, malathion became twice as lethal when combined with predatory stress. Similar synergistic interactions have been found with the insecticide carbaryl, suggesting that the synergy may occur in many carbamate and organophosphate insecticides. While malathion has the potential to kill amphibians and its presence is correlated with habitats containing declining populations, its actual role in amphibian declines is uncertain given the relatively low concentration in aquatic habitats.

**Keywords**—Predation Stress Synergy Frog Toad

## INTRODUCTION

Throughout the world, pesticides are used to improve crop production and human health by controlling undesirable plants and animals. In the United States, two billion kilograms of pesticides (active ingredient) are applied annually to forests, agricultural lands, homes, and gardens [1]. However, little is known about how these chemicals affect nontarget organisms. Amphibians are of particular concern because they appear to be declining on a global scale [2–5]. While amphibian declines are likely the result of a multitude of causes, including habitat destruction, disease, parasites, and introduced predators, recent studies suggest that some declines may be due to pesticides. Some declines are correlated with a proximity to greater amounts of agricultural lands (from which pesticides can be transported), and amphibians collected from these habitats have decreased acetylcholine esterase activity (a possible signal of carbamate and organophosphate chemicals) [6–9]. This suggests that pesticides could be playing a role in global amphibian declines.

For most pesticides, we have few data on the concentrations that are toxic to amphibians. Given the thousands of toxicology studies that have been conducted over the past several decades, the lack of data on amphibians is surprising. In aquatic systems, tests typically focus on fish and aquatic invertebrates but not on amphibians because pesticide registration does not require amphibian tests [10]. To evaluate the role that pesticides play in amphibian declines, we must conduct tests on amphibians. When amphibians are tested, studies frequently use the African clawed frog (*Xenopus laevis*). This species receives a disproportionate focus because we possess a tremendous amount of data on its biology and because *Xenopus* can be easily reared in the laboratory [11]. However, because

pesticide sensitivity can vary dramatically among different species, *Xenopus* may tell us little about the effects of pesticides on amphibians in general and even less about the effects of pesticides on North American amphibians.

In addition to the problems of few data and restricted taxonomic inference, most amphibian studies are conducted under unnatural conditions. Most studies determine the LC50 (the concentration that is estimated to kill 50% of a test population) for pesticides in 1- to 4-d experiments in the absence of food or any biotic and abiotic variation [10,12,13]. This is an efficient way of testing thousands of chemicals, but organisms in nature can be exposed to pesticides for longer periods of time, experience pesticides while foraging, and experience pesticides in combination with many biotic and abiotic stresses. Such stresses can have substantial impacts on a pesticide's toxicity [14–18]. For example, the pesticide carbaryl (commercial name: Sevin, Union Carbide, Research Triangle Park, NC, USA) can become up to 46 times more lethal when combined with the chemical cues emitted by aquatic predators [19,20]. Because most amphibian habitats contain predators, being exposed to pesticides in the presence of predator cues is the norm in nature [21–23]. The synergistic interaction between carbaryl and predator cues means that low concentrations of pesticides, once thought to be nonlethal, may be highly lethal in nature and potentially contribute to amphibian declines. However, our knowledge of these synergistic effects is based on only one pesticide (carbaryl). We need to determine whether synergistic interactions between pesticides and predator cues are common.

In this study, I examined how another common insecticide (malathion) affects amphibian mortality in both the presence and the absence of predator cues. I chose malathion for two reasons. First, both malathion and carbaryl are neurotoxins that inhibit acetylcholine esterase, so malathion might have

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similar synergistic interactions with predatory stress. Second, malathion is widely used to control insects, with a current focus on spraying wetlands for mosquito control to combat malaria and the West Nile virus [24,25]. I tested the effects of malathion and predator cues on the survival of six tadpole species (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*). While these species are not known to be experiencing population declines, using this diverse group of species may provide us with information as to the general toxicity of malathion. My objective was to determine LC50 estimates for each species and to test whether malathion and predator cues had synergistic effects.

#### Malathion background

Malathion is a broad-spectrum organophosphate insecticide that is widely used [1,24,25]. In the United States, 0.9 to 1.4 million kg of malathion are applied annually to more than 800,000 ha of cropland [1]; National Pesticide Use Database, www.ncfap.org/database/default.htm. Application rates for mosquito control range from 0.2 to 4.3 kg/ha (National Pesticide Use Database). For a wetland 30 cm deep that receives a direct overspray (a depth commonly inhabited by tadpoles), this translates to potential aquatic concentrations of 0.1 to 1.6 mg/L. The half-life depends on pH, ranging from 2 to 26 d under pH values of 8 to 6, respectively [26,27]. In surveys of aquatic habitats, malathion is typically present at concentrations of 0.001 to 0.6 mg/L [28,29]. While malathion is toxic to fish and aquatic invertebrates at low concentrations (<2 mg/L) [30–34], very little is known about its effect on amphibians [35–37].

#### METHODS

I raised the six species of tadpoles in the laboratory using randomized block designs. Because each species breeds at different times, I conducted six separate experiments. Each experiment included six malathion concentrations that spanned a range of natural to above-natural concentrations (0–20 mg/L) crossed with the presence or absence of predator cues. The 12 treatments were replicated four times, for a total of 48 experimental units per species. Experimental units were 10-L plastic tubs filled with 7.8 L of charcoal-filtered, ultraviolet-irradiated well water. All experiments were conducted at the University of Pittsburgh's Pymatuning Laboratory of Ecology (Linesville, PA, USA) under a 14:10-h light:dark cycle.

To obtain tadpoles, I collected newly oviposited eggs (1–15 egg masses per species) from ponds and wetlands surrounding Pymatuning Lake in northwestern Pennsylvania, USA. All eggs were hatched in aged well water to keep the tadpoles predator naive prior to the experiment. Soon after hatching (Gosner stage 25) [38], 10 tadpoles were placed into each tub. Tadpoles were fed ground fish flakes every 2 d at a ration of 18% of their body mass per day (initial mean mass  $\pm$  1 standard error: wood frogs =  $40 \pm 0.3$  mg, leopard frogs =  $25 \pm 2.0$  mg, toads =  $19 \pm 1.0$  mg, tree frogs =  $22 \pm 2.0$  mg, green frogs =  $14 \pm 2.0$  mg, bullfrogs =  $9 \pm 1.0$  mg). Halfway through the experiments, the ration was doubled to account for tadpole growth.

For the pesticide treatments, I added a commercial form of malathion. Its concentration (50.6% active ingredient) was quantified using high-pressure liquid chromatography analyses (Mississippi State Chemical Laboratory, Mississippi State,

MS, USA). For the six malathion concentrations (20, 10, 5, 1, and 0.1 mg/L), I added 312, 156, 78, 15.6, and 1.6  $\mu$ l of malathion solution. For the no-pesticide treatments, I added 312  $\mu$ l of water. To prevent the tub water from fouling, I changed the water every 4 d and reapplied the malathion after each water change (static renewal tests).

Predator cues were manipulated by adding one caged adult newt (*Notophthalmus viridescens*), a predator that coexists with all six prey species. The caged newts were fed approximately 100 mg of conspecific tadpoles every 2 d to produce the chemical cues that cause prey stress [21,22]. No-predator treatments contained empty cages that were lifted every 2 d to equalize disturbance across treatments.

I counted the number of surviving tadpoles each day and removed any dead animals. Midway through each experiment, I measured the temperature and pH of all tubs. The pH ranged from 7.8 to 8.2 among treatments. Temperature varied slightly among species, but the range for each species remained narrow among treatments (wood frogs = 18.7–19.0°C, leopard frogs = 18.6–18.7°C, toads = 20.1–20.3°C, tree frogs = 20.1–20.2°C, green frogs = 21.2–21.4°C, and bullfrogs = 21.2–21.5°C). Experiments were ended after 16 d (chronic tests).

#### Statistical analysis

I analyzed the survival in all experimental units using the proportion surviving in a tub as the response variable. The parametric assumption of homogeneous errors was not upheld (e.g., high concentrations of malathion caused 0% survival across all replicates). Therefore, I analyzed the data using a nonparametric approach that first ranked the data and then conducted a repeated measures analysis of variance. The two- and three-way block interactions with malathion concentrations and predator cues were never significant and, thus, were pooled with the error term. To estimate the LC50 values for each species, I used standard probit regression analyses.

#### RESULTS

Malathion had significant effects on the survival of all six amphibian species. Bullfrog survival was affected by malathion but not by predators or the malathion-by-predator interaction (Table 1 and Fig. 1). Bullfrogs experienced 0% survival with 5 to 20 mg/L of malathion. At 1.0 and 0.1 mg/L, bullfrog survival improved to 70 and 78%, respectively, but was still significantly lower than the controls (88%;  $p < 0.02$ ). The estimated LC50 for bullfrogs across both predator treatments was 1.50 mg/L.

In green frogs, survival was affected by malathion but not by predator cues or the malathion-by-predator interaction (Table 1 and Fig. 1). Green frogs exhibited 0% survival with 10 to 20 mg/L of malathion. At 5 mg/L, survival improved to 33%, but this was significantly lower than the controls ( $p < 0.00001$ ). At 1.0 and 0.1 mg/L, green frog survival was high (86–88%) and not different from the controls (91%;  $p > 0.3$ ). The estimated LC50 for green frogs across both predator treatments was 3.65 mg/L.

Gray tree frog survival was affected by both malathion and predator cues as well as the malathion-by-predator interaction (Table 1 and Fig. 1). Gray tree frogs experienced 0% survival with 10 to 20 mg/L of malathion. At lower concentrations, malathion became more deadly when combined with predator cues. At 5 mg/L, 42% of the tadpoles died when predator cues were absent, but 82% of the tadpoles died when predator cues were present (twice as lethal;  $p = 0.006$ ). Compared to the

Table 1. Analysis of variance results ( $p$  values) from the analyses of how six species of larval amphibians (in six separate experiments) survived under six concentrations of the pesticide malathion crossed with the presence or absence of predator cues over time. Species common names are abbreviated

Species	Bullfrog	Green frog	Tree frog	Toad	Leopard frog	Wood frog
Block	0.100	0.526	0.119	0.515	0.136	0.047
Malathion	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Predator	0.554	0.994	0.007	0.684	0.406	0.649
Predator $\times$ malathion	0.784	0.867	0.041	0.598	0.670	0.773
Time	<0.001	0.278	0.058	0.001	<0.001	0.943
Time $\times$ block	0.008	0.831	0.748	0.806	0.590	0.103
Time $\times$ malathion	<0.001	0.382	<0.001	<0.001	<0.001	<0.001
Time $\times$ predator	0.876	0.766	0.719	0.994	0.332	0.999
Time $\times$ malathion $\times$ predator	1.000	0.410	0.923	0.998	0.332	0.270

controls, mortality was similar when predators were absent ( $p = 0.067$ ) but higher when predator cues were present ( $p = 0.005$ ). At 1 and 0.1 mg/L, survival tended to be lower with predator cues, but it was not significant ( $p = 0.131$  and  $0.228$ , respectively). Tree frog survival at 1 and 0.1 mg/L was not different from the controls (71%;  $p > 0.05$ ). The estimated LC50 for gray tree frogs was 4.13 mg/L without predator cues and 2.00 mg/L with predator cues.

In toads, survival was affected by malathion but not by predators or the malathion-by-predator interaction (Table 1 and Fig. 1). Survival was 0% in 10 to 20 mg/L of malathion. Survival improved to 76% in 5 mg/L but remained significantly lower than the controls ( $p < 0.001$ ). At 1.0 and 0.1 mg/L, survival was high (96–99%) and not different from the control

tadpoles ( $p > 0.8$ ). The estimated LC50 for toads across both predator treatments was 5.9 mg/L.

Leopard frog survival was affected by malathion but not by predators or the malathion-by-predator interaction (Table 1 and Fig. 1). They experienced 0% survival in 5 to 20 mg/L of malathion. At 1.0 and 0.1 mg/L, survival was high (96–98%) and not different from the control treatments (99%;  $p > 0.1$ ). The estimated LC50 across both predator treatments was 2.40 mg/L.

Wood frog survival was affected by malathion but not by predators or the malathion-by-predator interaction (Table 1 and Fig. 1). Survival was 0% in 10 to 20 mg/L of malathion and only 5% in 5 mg/L of malathion. Survival improved at 1.0 and 0.1 mg/L (51 and 65%, respectively) but remained lower

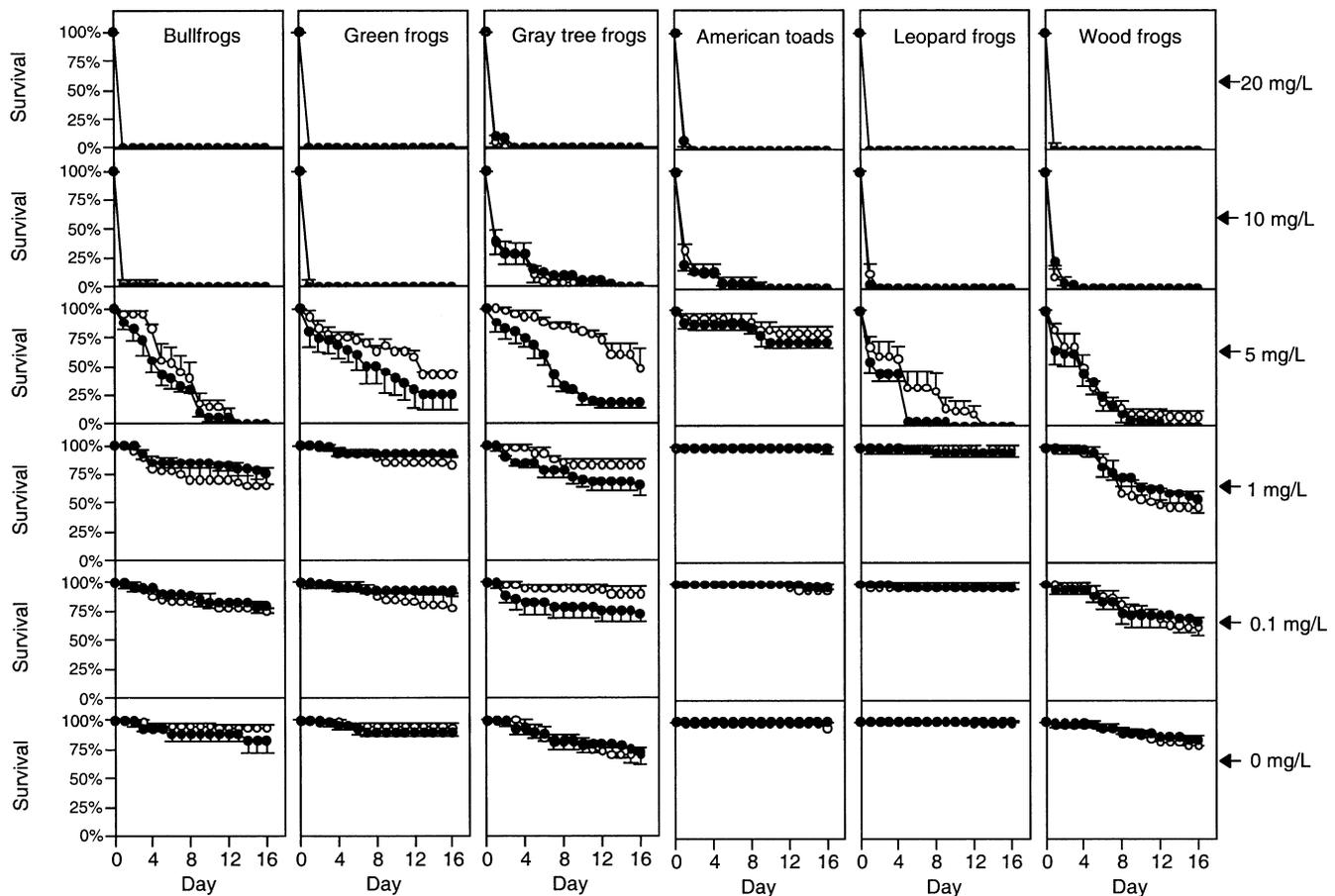


Fig. 1. Survival (mean  $\pm$  1 standard error) of tadpoles when exposed to a factorial combination of predator cues (absent = open symbols; present = closed symbols) crossed with six concentrations of malathion.

than the control tadpoles (83%;  $p < 0.03$ ). The estimated LC50 for wood frogs across both predator treatments was 1.25 mg/L.

## DISCUSSION

The experiments allowed for LC50 estimates for each of the six tadpole species and revealed synergistic effects of predator cues and malathion in one of the species. The LC50 estimates were highest for toads (5.9 mg/L) and green frogs (3.7 mg/L), intermediate for leopard frogs (2.4 mg/L) and gray tree frogs (2.0–4.1), and lowest for bullfrogs (1.5 mg/L) and wood frogs (1.3 mg/L). Such species-specific patterns in pesticide sensitivity matches the pattern of amphibian declines in different parts of the world; in a given habitat, some species decline while other species do not [3–5,9]. Given the maximum estimate of malathion concentration in aquatic habitats 30 cm deep (1.6 mg/L), the probit analyses estimated that 4 to 55% of each species would die from malathion exposure. At the concentrations observed in aquatic habitats (0.001–0.6 mg/L) [28,29], the probit analyses estimated that malathion should kill 2 to 35% of each species.

By running the experiments for 16 d rather than the more traditional 1- to 4-d tests, one arrives at a very different perspective concerning the toxicity of malathion to tadpoles. For example, if we had tested bullfrogs for only 3 d without predators, we would have concluded that 5 mg/L caused only 5% mortality. However, when exposed for 16 d, malathion caused 100% mortality. Similarly, a 4-d test with wood frogs at 1 mg/L would cause only 5% mortality, but a 16-d test would cause 52% mortality. While it is expected that longer duration tests should cause higher mortality [10], it is not obvious that the differences should be this large.

Studies of larval amphibian responses to malathion are limited. Rosenbaum et al. [35] found that toad embryos (*Bufo arenarum*) were very resistant to malathion (LC50<sub>s,d</sub> = 42 mg/L). In contrast, Fordham et al. [35] found that bullfrog larvae began to suffer significant mortality when malathion concentrations exceeded 2.5 mg/L (a 28-d static renewal test). The latter study is in general agreement with the bullfrogs in the present study, although I also observed a small (10%) decrease in survival even at 0.1 mg/L. It is perhaps surprising that few studies have compared LC50 values. However, current regulations do not require tests on amphibians. For example, a query of the federal toxicity database (<http://toxnet.nlm.nih.gov>) for LC50 estimates illustrates the taxonomic bias: 4 bird studies, 19 invertebrates studies, 39 fish studies, and no amphibian studies. Thus, current estimates of pesticide risk to amphibians frequently must be based on the toxicity to fish and aquatic invertebrates [10], yet the toxicity to amphibians may be quite different than the toxicity to fish and aquatic invertebrates.

Malathion is highly toxic to many fish and aquatic invertebrates. The LC50 values (96-h tests) for fish are wide ranging, but frequently they are quite low, including tilapia (*Oreochromis niloticus*) = 2.0 mg/L, killifish (*Oryzias latipes*) = 1.8 mg/L, rainbow trout (*Salmo gairdneri*) = 0.1 mg/L, bluegill (*Lepomis macrochirus*) = 0.1 mg/L, and walleye (*Stizostedion vitreum*) = 0.06 mg/L [30,33,39]. The LC50 values (72- to 96-h tests) also are typically low for aquatic invertebrates, including crayfish (*Orconectes nais*) = 0.18 mg/L, grass shrimp (*Palaemonetes pugio*) = 0.013 mg/L, stoneflies (*Pteronarcys californica*) = 0.01 mg/L, amphipods (*Gammarus palustris*) = 0.005 mg/L, and cladocerans (*Moina macrocarpa*) = 0.005 mg/L [32,34,39,40]. These studies high-

light the difficulty of extrapolating LC50 values to amphibians. They also make it clear that malathion has the potential to reduce a great deal of aquatic biodiversity.

The LC50 values are often treated as reliable estimates of toxicity to an organism under natural conditions. However, in one of the six species tested (gray tree frogs), predator cues made malathion at intermediate concentrations (5 mg/L) twice as deadly. Synergistic interactions between pesticides and predator cues were first discovered in carbaryl (a broad-spectrum carbamate insecticide). In the most extreme case, predator cues made carbaryl 46 times more deadly [19,20]. Most larval amphibians in North America live with aquatic predators, and these predators commonly cause stress in tadpoles; thus, most amphibians exposed to pesticides are already under predatory stress [21,22]. Synergistic effects of predator cues have been previously observed in three of six species exposed to carbaryl (gray tree frogs, bullfrogs, and green frogs [20]) but in only one of the six species exposed to malathion (gray tree frogs). This suggests that although both insecticides operate by inhibiting acetylcholine esterase, the synergies between pesticides and predator cues can be quite pesticide and species specific.

The mechanism that underlies the synergy between pesticides and predator cues is unknown. Previous studies have ruled out the possibility that the synergy occurs because predators affect the pH of the water or alter the concentrations of ammonia or oxygen [19,20]. The most probable mechanism is that the synergy is the result of multiple stressors in which the stress of the pesticide becomes harmful only when combined with the stress of predator cues. To determine the mechanism, we need to examine amphibian physiology and the associated stress hormones in each environmental combination.

These studies underscore the importance of examining pesticides under more natural conditions. Several investigators have demonstrated that pesticides become more toxic under different abiotic environments, including changes in temperature, pH, and ultraviolet-B radiation [14–16]. However, the importance of biotic variation in natural systems is much less appreciated. For example, competition can make carbaryl either more or less lethal to Woodhouse's toads (*Bufo woodhousii*) and gray tree frogs [17,18]. Further, malathion can make Woodhouse's toads more susceptible to the bacteria that cause red-legged disease (*Aeromonas hydrophila*) [37]. In light of the recent discovery of synergies between pesticides and predator cues, these studies suggest that the abiotic and biotic variation that frequently occurs in nature can have important impacts on our estimates of pesticide toxicity. Therefore, it is critical that we include this ecological reality in our studies of toxicology.

The causes of declining amphibian populations are numerous and include habitat loss, introduced predators, and disease [2–5]. It is becoming increasingly clear that pesticides may also be playing a role in these declines [6–9]. Globally common insecticides, such as carbaryl and malathion (and perhaps other carbamate and organophosphate insecticides), can enter aquatic habitats at concentrations that can be lethal to many species of amphibians, particularly when combined with other natural stressors. This evidence places decision makers in the difficult position of balancing the need to control pests, including the mosquitoes that carry malaria and West Nile virus, with the need to protect amphibians.

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