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GROWTH AND SURVIVAL OF FIVE AMPHIBIAN SPECIES EXPOSED TO COMBINATIONS OF PESTICIDES

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Abstract—The global decline of amphibians has sparked interest in the role that pesticides may play. Pesticides in nature typically exist in combinations, but given the vast number of chemicals used, most toxicological experiments necessarily have examined one pesticide at a time. I examined how four commercial formulations of pesticides (diazinon, carbaryl, malathion, and glyphosate) affected the survival and growth of five larval amphibian species (*Rana pipiens*, *R. clamitans*, *R. catesbeiana*, *Bufo americanus*, and *Hyla versicolor*) when alone (at 1 or 2 mg/L of active ingredient) and in pairwise combinations (1 mg/L of each pesticide). At 1 mg/L, the pesticides reduced survival in 5% of the 20 species–pesticide comparisons and reduced growth in 35% of the comparisons. At 2 mg/L, the pesticides had more widespread effects, reducing survival in 35% of the 20 species–pesticide comparisons and reducing growth in 70% of comparisons. Combined pesticides occasionally caused lower survival and growth than either pesticide alone, but the effects were never larger than the more deadly of the two pesticides alone at 2 mg/L. This suggests that the impact of combining these four pesticides is similar to that predicted by the total concentration of pesticides in the system.

Keywords—Tadpole Synergy Combined pesticides Growth Survival

INTRODUCTION

The global decline of amphibians has been debated for the past decade as biologists have struggled to collect the long-term data necessary to establish the evidence. The most recent evidence suggests that some species are declining while others are not [1–3]. Although numerous causes of amphibian declines have been proposed, including habitat loss, disease, climate change, and introduced predators and competitors [1,4–6], there is growing evidence that pesticides also may be playing a role [6–9].

Because of this interest in amphibian decline, there has been an explosion of interest in examining how a plethora of different pesticides affects amphibians. This work has included numerous experiments to estimate the LC50 values of pesticides (the concentration estimated to kill 50% of a population within a given amount of time [10–15]). Subsequent experiments have examined how these lethal effects of pesticides can vary under different biotic and abiotic conditions [16–20]. Additional experiments have documented that even sublethal concentrations can have important effects on the behavior, life history, and reproduction of amphibians [21–22].

This growing body of research has taught us a great deal about the impacts that a given pesticide can have on amphibians under a wide variety of conditions. However, the vast majority of this work has only examined one pesticide at a time. This is in stark contrast to patterns in natural amphibian habitats in which investigators have found combinations of pesticides [8–9,23–24]. While the impact of combined pesticides has received empirical attention in other systems [25–27], we know little about the impacts of combined pesticides on amphibians [28].

In this study, I examined the effects of separate and combined pesticides on the survival and growth of five amphibian species: Leopard frogs (*Rana pipiens*), green frogs (*R. clamitans*), bullfrogs (*R. catesbeiana*), American toads (*Bufo americanus*), and gray tree frogs (*Hyla versicolor*). I used three different broad-spectrum insecticides, diazinon (Monsanto, St. Louis, USA), malathion (Gro Tec, Madison, GA, USA), and carbaryl (commercial name Sevin®; Tech Pac, Lexington, KY, USA), that inhibit acetylcholine esterase in animals and one broad-spectrum herbicide, glyphosate (commercial names Roundup®, Rodeo®; Monsanto), that inhibits the synthesis of essential amino acids in plants. All four pesticides are commonly used worldwide and are among the most commonly used pesticides in the United States [29].

Four pesticides were used alone at low concentrations (1 mg/L active ingredient), alone at high concentrations (2 mg/L), and in six pairwise combinations (1 mg/L of each pesticide combined). Based on the null assumption that pesticide effects are additive, I tested the three hypotheses. First, when each pesticide is present alone, high concentrations will cause lower survival and growth than low concentrations. Second, when two pesticides are combined, survival and growth will be lower than with either pesticide alone at low concentrations. Third, when two pesticides are combined, survival and growth will be intermediate to either pesticide alone at high concentration (assuming that the two pesticide cause different effects when alone).

METHODS AND MATERIALS

Because the five species breed at different times of the year, I conducted five separate experiments. Each experiment was a randomized block design with each block (laboratory shelves at different heights) containing 15 treatments. The treatments were replicated four times (four spatial blocks represented by four shelf heights) for a total of 60 experimental units for each of the five species. The experimental units were 10-L plastic tubs filled with 8 L of charcoal-filtered, ultraviolet-irradiated well water. The first treatment was the control, in which 36 μl of water were added. Four treatments contained 1 mg/L

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(active ingredient) of Sevin, diazinon, malathion, or Roundup. Four treatments contained 2 mg/L (active ingredient) of carbaryl (Sevin), diazinon, malathion, or glyphosate (Roundup). The final six treatments contained the six possible pairwise combinations of the four pesticides (with each pesticide at 1 mg/L). I used commercial formulations of all pesticides whose concentrations were confirmed using high pressure liquid chromatography (carbaryl = 22.3%, diazinon = 22.4%, malathion = 50.6%, and glyphosate = 25.2%; Mississippi State Chemical Laboratory, Mississippi State, MS, USA). The four pesticides also contain inert ingredients that are not disclosed (and often are trade secrets), although Roundup contains a POEA surfactant (polyethoxylated tallowamine) to help permeate leaf cuticles [14]. Based on these concentrations, I added 36 μl of carbaryl, 36 μl of diazinon, 16 μl of malathion, and 32 μl of glyphosate to achieve 1 mg/L nominal concentrations. These nominal concentrations were not tested separately following each pesticide's application.

The tadpoles for each experiment were collected as newly oviposited eggs (using 3–10 masses per species) that were hatched in outdoor wading pools containing aged well water. After hatching (Gosner stage 25 [30]), groups of ten tadpoles were added to each experimental tub. Initial mean tadpole masses (±1 standard error), after being blotted free of water, were as follows: Leopard frogs = 95 ± 4 mg, American toads = 56 ± 5 mg, gray tree frogs = 73 ± 8 mg, green frogs = 28 ± 2 mg, bullfrogs = 23 ± 2 mg. Tadpoles were fed a constant 9% daily per capita ration (equal across all tubs) of ground fish flakes every 2 d. This ration was doubled midway through the experiment to account for tadpole growth. To prevent the water from fouling, water was changed and the pesticide treatments were reapplied (i.e., chronic, static renewal tests) every 4 d. I quantified survival every day and removed any dead tadpoles. During the leopard frog and American toad experiments, I measured water quality parameters and found no difference in rearing conditions (water temperature = 20.4–20.9°C, pH = 8.0, dissolved oxygen 2.8–3.5 mg/L, and ammonia = 2.8–4.2 mg/L).

Each experiment lasted 16 d. At the end of each experiment, I counted the final number of survivors in each tub and the growth rate of those survivors (final mass − initial mass)/16 d). Using the survival and mean growth of each tub as my response variables, I analyzed the data using analyses of variance. The survival data were heteroscedastic (survival was often very high with little variation), so I first ranked the survival data with equal ranks given to tubs with the same survival. When there were significant univariate effects, I compared treatment means using Fisher's test.

RESULTS

Because of the large number of species and treatments, I first report the effects of the pesticides on tadpole survival and then report the effects of the pesticides on tadpole growth. Within each of these sections, I examine the effects of each pesticide alone at 1 mg/L and 2 mg/L compared to the control treatment. Next, I compare the impact of combined pesticides to each pesticide alone at 1 mg/L. Finally, I compare the impact of combined pesticides to each pesticide alone at 2 mg/L. For ease of reference to each pesticide composing a pair, I use the term weaker pesticide to refer to the pesticide that causes less mortality or growth reduction when alone and the term stronger pesticide to refer to the pesticide that causes greater mortality or growth reduction when alone. At the end of each section,

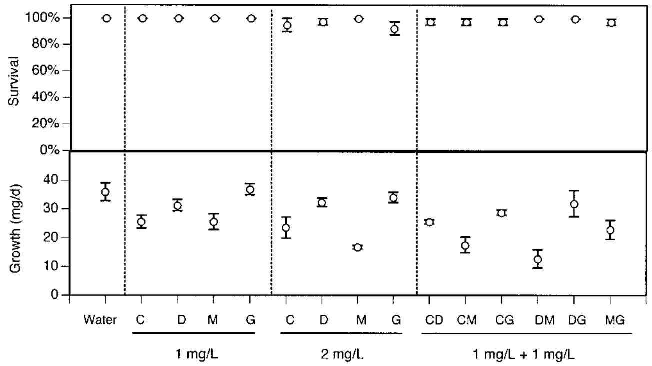

Fig. 1. The survival and growth (mean ± 1 standard error) of leopard frog tadpoles when exposed to different concentrations of separate and combined pesticides (n = 4 for all treatments). Treatments are abbreviated as follows: C = carbaryl, D = diazinon, M = malathion, G = glyphosate.

I provide a summary of the results pooled across all five species. The common occurrence of block effects can be attributed to different shelf positions in the experiments.

Survival

The different pesticides occasionally impacted the survival of the five tadpole species. In leopard frogs, there was no effect of block (p = 0.525) or treatment (p = 0.663) on tadpole survival (Fig. 1). Thus, pesticides present separately and in combination had no effect on leopard frog survival.

In American toads, survival was affected by both block (p = 0.017) and treatment (p = 0.017; Fig. 2). At 1 mg/L, survival was not reduced with any of the pesticides ($p > 0.2$). At 2 mg/L, survival was reduced with malathion and glyphosate ($p \leq 0.03$). Combined pesticides caused similar lethality to each pesticide alone at 1 mg/L in all comparisons ($p > 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides were equally lethal in all six comparisons ($p > 0.05$). However, compared to the stronger pesticide alone at 2 mg/L, combined pesticides were less lethal in four comparisons ($p < 0.04$) and equally lethal in two comparisons ($p > 0.05$).

In gray tree frogs, survival was affected by treatment (p = 0.009) but not by block (p = 0.350; Fig. 3). At 1 mg/L, survival was unaffected by any of the pesticides ($p > 0.1$). However, at 2 mg/L, there was marginally lower survival with diazinon (p = 0.055). Compared to the weaker pesticide alone at 1 or

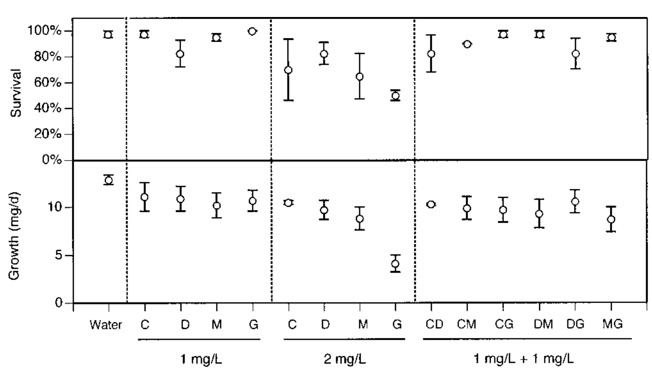

Fig. 2. The survival and growth (mean ± 1 standard error) of American toad tadpoles when exposed to different concentrations of separate and combined pesticides (n = 4 for all treatments). Treatments are abbreviated as follows: C = carbaryl, D = diazinon, M = malathion, G = glyphosate.

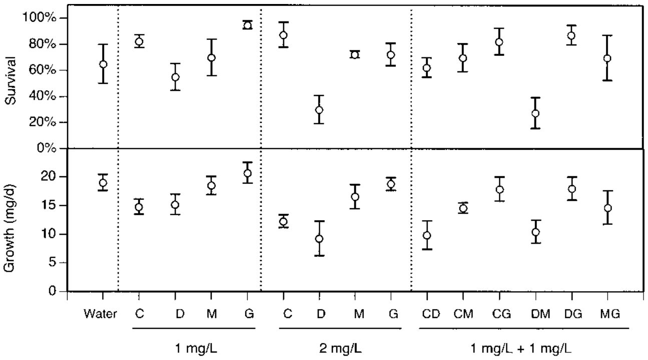

Fig. 3. The survival and growth (mean ± 1 standard error) of gray tree frog tadpoles when exposed to different concentrations of separate and combined pesticides ($n = 4$ for all treatments). Treatments are abbreviated as follows: C = carbaryl, D = diazinon, M = malathion, G = glyphosate.

2 mg/L, combined pesticides were equally lethal in five comparisons ($p > 0.05$) and more lethal in one comparison (diazinon plus malathion; $p = 0.027$ and $p = 0.038$, respectively). Compared to the stronger pesticide alone at 1 or 2 mg/L, combined pesticides were equally lethal in five comparisons ($p > 0.05$) and less lethal in one comparison (diazinon plus glyphosate; $p = 0.025$ and $p = 0.001$, respectively).

In green frogs, survival was affected by treatment ($p < 0.001$) but not by block ($p = 0.483$; Fig 4). At 1 mg/L, only diazinon caused significant mortality ($p = 0.019$). At 2 mg/L, both diazinon and glyphosate caused significant mortality ($p \leq 0.001$). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides were equally lethal in two comparisons ($p > 0.05$) and more lethal in four comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides were equally lethal in all six comparisons ($p > 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides were equally lethal in four comparisons ($p > 0.05$) but more deadly in two comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides were less lethal in three comparisons ($p = 0.001$) and equally lethal in three comparisons ($p > 0.05$).

In bullfrogs, survival was affected by treatment ($p < 0.001$) but not by block ($p = 0.668$). At 1 mg/L, none of the pesticides reduced survival ($p > 0.07$). However, at 2 mg/L, survival was reduced with diazinon, malathion, and glyphosate ($p \leq 0.001$). Compared to the weaker pesticide alone (at 1 mg/L), combined pesticides were equally lethal in four comparisons ($p > 0.05$) and more lethal in two comparisons (diazinon plus malathion, diazinon plus glyphosate; $p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides were equally lethal in four comparisons ($p > 0.05$) and more lethal in two comparisons (diazinon plus malathion, diazinon plus glyphosate; $p < 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides were equally lethal in all six comparisons ($p > 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides were less lethal in two comparisons ($p = 0.001$) and equally lethal in four comparisons ($p > 0.05$).

By pooling mortality across all five species, we can develop a general sense of the lethality from these pesticides. At 1 mg/L, the pesticides caused significant mortality in only 5% of the comparisons (1/20). At 2 mg/L the pesticides caused significant mortality in 35% of the comparisons (7/20). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides were equally lethal in 77% of the comparisons (23/30) and more lethal in 23% of the comparisons (7/30). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides were less lethal in 3% of the comparisons (1/30), equally lethal in 87% of the comparisons (26/30), and more lethal in 10% of the comparisons (3/30). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides were equally lethal in 90% of the comparisons (27/30) and more lethal in 10% of the comparisons (3/30). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides were less lethal in 33% of the comparisons (10/30), equally lethal in 67% of comparisons (20/30), and more lethal in 0% of comparisons (0/30).

Growth

The different pesticide environments also impacted tadpole growth. In leopard frogs, growth was affected by both treatment ($p < 0.001$) and block ($p < 0.001$; Fig. 1). At 1 mg/L and 2 mg/L, growth was reduced with carbaryl and malathion ($p < 0.001$). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides caused similar growth in one comparison ($p > 0.05$) and lower growth in five comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides caused similar growth in four comparisons ($p > 0.05$) and lower growth in two comparisons ($p < 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in two comparisons ($p > 0.05$) and lower growth in four comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in one comparison ($p > 0.05$) and similar growth in five comparisons ($p < 0.05$).

In American toads, growth was affected by both treatment ($p < 0.001$) and block ($p < 0.001$; Fig. 2). At 1 mg/L, growth was reduced with malathion ($p = 0.040$). At 2 mg/L, growth was reduced with diazinon, malathion, and glyphosate ($p < 0.02$). Compared to either pesticide alone at 1 mg/L, combined pesticides caused similar growth in all comparisons ($p > 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in all six comparisons ($p > 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in three comparisons ($p > 0.05$) and similar growth in three comparisons ($p < 0.05$).

In gray tree frogs, growth was affected by treatment ($p = 0.001$) but not block ($p = 0.452$; Fig. 3). At 1 mg/L, growth was not affected by any of the pesticides ($p < 0.001$). However,

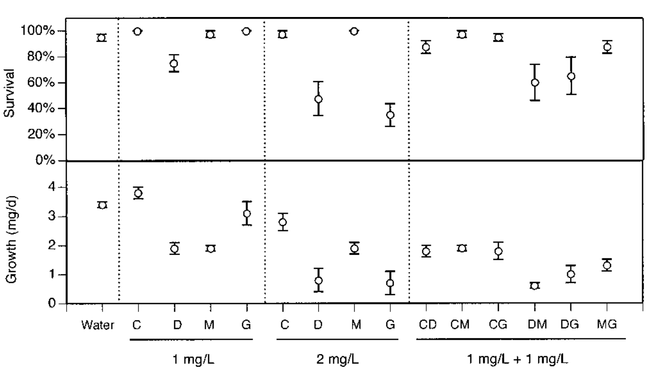

Fig. 4. The survival and growth (mean ± 1 standard error) of green frog tadpoles when exposed to different concentrations of separate and combined pesticides ($n = 4$ for all treatments). Treatments are abbreviated as follows: C = carbaryl, D = diazinon, M = malathion, G = glyphosate.

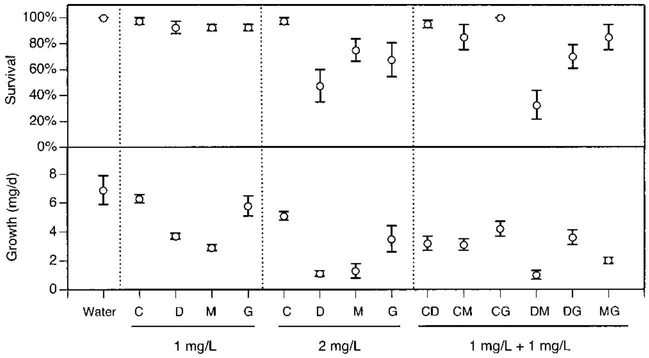

Fig. 5. The survival and growth (mean ± 1 standard error) of bullfrog tadpoles when exposed to different concentrations of separate and combined pesticides (n = 4 for all treatments). Treatments are abbreviated as follows: C = carbaryl, D = diazinon, M = malathion, G = glyphosate.

at 2 mg/L, growth was reduced with carbaryl and diazinon ($p < 0.02$). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides caused similar growth in four comparisons ($p > 0.05$) and lower growth in two comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides caused similar growth in all six comparisons ($p > 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in five comparisons ($p > 0.05$) and lower growth in one comparison ($p < 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in two comparisons ($p > 0.05$) and similar growth in four comparisons ($p < 0.05$).

In green frogs, growth was affected by both treatment ($p < 0.001$) and block ($p < 0.001$; Fig. 4). At 1 mg/L, growth was reduced with diazinon and malathion ($p < 0.001$). However, at 2 mg/L, growth was reduced with diazinon, malathion, and glyphosate ($p < 0.001$). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides always caused lower growth ($p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides caused similar growth in three comparisons ($p > 0.05$) and lower growth in three comparisons ($p < 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in two comparisons ($p > 0.05$) and lower growth in four comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in two comparisons ($p > 0.05$) and similar growth in four comparisons ($p < 0.05$).

In bullfrogs, growth was affected by treatment ($p < 0.001$) but not by block ($p = 0.818$; Fig. 5). At 1 mg/L, growth was reduced with diazinon and malathion ($p < 0.001$). At 2 mg/L, growth was reduced with all four pesticides ($p < 0.001$). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides always caused lower growth ($p < 0.05$). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides caused similar growth in five comparisons ($p > 0.05$) and lower growth in one comparison ($p < 0.05$). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in four comparisons ($p > 0.05$) and lower growth in two comparisons ($p < 0.05$). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in four comparisons ($p > 0.05$) and similar growth in two comparisons ($p < 0.05$).

By pooling the growth results across all five species, we can develop a more general sense of growth effects of combined pesticides. At 1 mg/L, the pesticides caused significantly lower growth in 35% of the comparisons (7/20). At 2 mg/L the pesticides caused significantly lower growth in 70% of the comparisons (14/20). Compared to the weaker pesticide alone at 1 mg/L, combined pesticides caused similar growth in 37% of the comparisons (11/30) and lower growth in 63% of the comparisons (19/30). Compared to the stronger pesticide alone at 1 mg/L, combined pesticides caused similar growth in 80% of the comparisons (24/30) and lower growth in 20% of the comparisons (6/30). Compared to the weaker pesticide alone at 2 mg/L, combined pesticides caused similar growth in 63% of the comparisons (19/30) and lower growth in 37% of the comparisons (11/30). Compared to the stronger pesticide alone at 2 mg/L, combined pesticides caused higher growth in 40% of the comparisons (12/30), similar growth in 60% of the comparisons (18/30), and lower growth in 0% of the comparisons.

DISCUSSION

The experiments demonstrated that combined pesticides can sometimes have larger effects on amphibian survival and growth than either pesticide alone. When present alone at 1 mg/L, the four pesticides rarely had any negative impact on tadpole survival. However, at 2 mg/L, several of the pesticides caused significant mortality and the mortality patterns were both pesticide- and species-specific. Carbaryl did not cause mortality in any of the species; diazinon caused mortality in gray tree frogs, green frogs, and bullfrogs; malathion caused mortality in American toads and bullfrogs; and glyphosate caused mortality in American toads, green frogs, and bullfrogs. These lethal effects are consistent with previous toxicity studies on these species [13,20]. Combinations of pesticides occasionally caused greater death than either of the pesticides alone at 1 mg/L. Combining 1 mg/L of each pesticide caused no further reduction in the survival of leopard frogs, American toads, gray tree frogs, or green frogs. However, in bullfrogs, two combinations of pesticides caused lower survival than each pesticide alone (at 1 mg/L). This suggests that exposure to multiple pesticides in nature [8,24] can be more lethal than predicted from toxicology studies involving one pesticide at a time. However, the current study also included doubled concentrations of each pesticide alone to examine whether the combined pesticide effect was more likely due to exposure to a particular combination of pesticides used or exposure to a higher total concentration of pesticides. In no case did combinations of pesticides cause lower survival than doubling the concentration of the more deadly pesticide. Thus, the effects of combined pesticides on tadpole survival simply could be the effect of experiencing a greater total amount of pesticide and not the effect of a particular pesticide combination.

Impacts of pesticides on tadpole growth were much more prevalent than the impacts on tadpole survival. For example, pesticides at 1 mg/L affected survival in 5% of the comparisons, but they affected growth in 35% of the comparisons. Similarly, pesticides at 2 mg/L affected survival in 35% of the comparisons, but they affected growth in 70% of the comparisons. This difference in lethal and nonlethal impacts was obvious especially in leopard frogs; there were no effects of the pesticides on leopard frog survival but effects on growth were widespread. These results concur with past studies of pesticide effects on amphibians in which tadpoles often survive exposure to pesticides but still experience altered growth and behavior [10,12,19,21].

Similar to the survival effects, combining pesticides occasionally resulted in lower growth than either of the pesticides alone. Combining 1 mg/L of each pesticide caused no further reduction in growth for American toads and gray tree frogs but did cause further reductions in growth for leopard frogs, green frogs, and bullfrogs. Lower growth has long-term implications to amphibian fitness. First, for species that live in ephemeral ponds and wetlands that dry each year, slower growth can result in the death of an entire population's cohort if tadpoles are not able to metamorphose in time [31]. Moreover, even if tadpoles are able to metamorphose before their habitat dries, slower growth and a smaller body size at metamorphosis has additional long-term fitness effects including reduced survival, smaller size at maturity, and lower egg production by females [31–35]. Thus, even when combined pesticides do not have immediate impacts on tadpole survival, the impacts on tadpole growth may affect long-term population dynamics of amphibians.

By including the 2 mg/L pesticide concentrations, we can determine whether these combined pesticide effects on growth were more likely the impact of combinations of pesticides or the impact of a higher total concentration of pesticides. In every case, the reduced growth caused by combined pesticides was not more extreme than the reduced growth caused by doubling the concentration of the stronger pesticide alone, suggesting that growth can be predicted from the total concentration of pesticides. Although this conclusion probably is not universal for all pesticides, it certainly is consistent with toxicity studies with other taxa [25–27].

Given the nearly 80,000 registered chemicals that need to be tested, it is a substantial challenge to understand how each of these chemicals affects a wide variety of organisms. Even more daunting is to determine how combinations of these chemicals affect organisms. Thus, it is no wonder that we know relatively little about how combination pesticides affect larval amphibians. Exposure to combinations of pesticides is a common scenario for larval amphibians in nature [8–9,23–24] and many amphibians are declining [2], so we need to address this challenge rapidly. The results of the current study suggest that combinations of commercial pesticide formulations can cause lower survival and growth of tadpoles of many different species (either due to their active ingredients or their additional inert ingredients), but these combined effects can be predicted from the total pesticide concentrations. Clearly, we need more investigations into combined pesticide effects to determine if these conclusions can be applied generally to other amphibian species.

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