EFFECTS OF CARBARYL ON GREEN FROG (RANA CLAMITANS) TADPOLES: TIMING OF EXPOSURE VERSUS MULTIPLE EXPOSURES

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Abstract—The majority of studies on pesticide impacts have evaluated the effects of single exposures. However, multiple exposures to a pesticide may be more prevalent. The objective of our study was to determine how multiple exposures versus single exposure at different times during development affected survival to metamorphosis, tadpole survival, tadpole mass, and tadpole developmental stage of green frog (Rana clamitans) tadpoles reared at low and high density in outdoor cattle tank ponds. Tadpoles were exposed to carbaryl zero, one, two, or three times at 14-d intervals. We applied single doses of carbaryl at one of three times, specifically during early, mid, or late development. Overall, we found that multiple exposures had a greater impact than single exposures during development. More individuals reached metamorphosis in ponds exposed to multiple doses of carbaryl compared with controls, indicating that the presence of carbaryl stimulated metamorphosis. The presence of carbaryl in the aquatic environment also resulted in more developed tadpoles compared with controls. Tadpoles in control ponds did not reach metamorphosis and were less developed than individuals exposed to carbaryl; this effect indicates that, under ideal conditions, green frogs could overwinter in ponds so that greater size could be attained before metamorphosis in the following spring or summer. Our study demonstrated the importance of including realistic application procedures when evaluating the effects of a pesticide and that multiple exposures to a short-lived pesticide are more likely to affect an amphibian population.

Keywords—Carbaryl Pesticide Anuran Amphibian decline Rana clamitans

INTRODUCTION

Amphibian populations are experiencing worldwide declines, which may be caused by contaminants, disease, ultraviolet radiation, habitat destruction, exotic or invasive species, or a combination of these factors [1]. Pesticides are particularly relevant because they have been correlated with amphibian population declines in some areas [2]; however, direct causality has not been demonstrated. Therefore, it is relevant to assess the direct and indirect role of contaminants in amphibian population declines. Amphibian metamorphosis is affected by the interaction of natural factors like pond hydroperiod, competition, and predation [3–5] in addition to widespread anthropogenic stresses like chemical contaminants [6]. Experimental studies addressing the effects of pesticides in realistic environmental settings with realistic exposure concentrations and frequency are necessary to understand the effects that pesticides may have on nontarget species like amphibians.

Toxicological studies with amphibians are relatively limited in number [7]. However, amphibians may be particularly vulnerable to pesticides given that they favor habitats that may be exposed to pesticides, they have limited mobility by which to avoid exposure, and many amphibians have complex life cycles that may lead to exposure in both aquatic and terrestrial environments [8]. The complexity of the amphibian life cycle indicates that more subtle endpoints (e.g., size, time, and survival to metamorphosis) may be critical measures in determining the susceptibility of amphibians to contaminants, particularly given that environmental levels of many pesticides may not be lethal to amphibians [6].

Consideration of the effects of pesticides on food-web dynamics, which is possible in mesocosm field studies, will increase our ability to predict contaminant effects in nature. Recently, a number of studies have examined the impacts of contaminant exposure on metamorphosis (e.g., [9–15]). In addition to measuring relevant biological endpoints and creating a realistic ecological context, it is also necessary to mimic field application practices. Although agricultural pesticides are often applied repetitively throughout a growing season, standard test protocols typically do not include multiple exposures [16,17]. Therefore, many standard tests with pesticides may represent unrealistic chemical circumstances by assuming organisms will be exposed to a single, acute dose.

Exposure to a pesticide multiple times may be particularly germane for short-lived pesticides, which are more likely to be used repetitiously; therefore, short-lived pesticides may culminate in multiple chemical exposures. Of course, repeated exposures may or may not elicit a response greater than a single exposure. Sufficient recovery time between exposures may minimize the negative effects of repeated exposures [18]. The family of a pesticide may also influence how single versus multiple exposures affect organisms and their communities. For instance, carbamates and organophosphates both result in acetylcholinesterase inhibition; however, inhibition from carbamates is reversible while inhibition from organophosphates is considered irreversible [18,19]. Kallander et al. [18] found that, given enough recovery time, midges (Chironomus riparius) could recover from exposure to carbamates but not from exposure to organophosphates. Multiple exposures, however, may alter community dynamics more dramatically than a single exposure in some cases. For instance, Hazazota and Yasuno [20] found that repeated applications of the carbamate insecticide carbaryl changed the community structure of zoo-
plankton, whereas the community recovered from a single dose. Nevertheless, multiple exposures may not elicit an effect greater than a single exposure. Studies with fish [16] and amphibians [21] have found that exposure to multiple doses of insecticides had no cumulative effects.

The time that exposure to a pesticide occurs during development influences the effect on amphibians [10,21,22]; therefore, timing of exposure may be a component of an organism’s response to multiple exposures. Boone et al. [23] found that amphibian metamorphosis was significantly greater in high-density (i.e., high-competition) ponds exposed to carbaryl three times than in ponds exposed to carbaryl zero, one, or two times. However, it is unclear whether metamorphosis was stimulated from multiple exposures, the time during development when exposure occurred, or a combination of the two. Exposure to chemicals late in larval development has been found to reduce survival [9,22] and suggests that amphibians at later stages of metamorphosis are more sensitive to environmental perturbations. The effects of multiple exposures versus the timing of a single exposure, which are often confounded in multiple-exposure studies, have not been explicitly tested.

The insecticide carbaryl (the active ingredient in Sevin [Ortho, Columbus, OH, USA]) is a carbamate acetylcholinesterase inhibitor that is a widely used agricultural chemical. We elected to use carbaryl in our study because it has been used in numerous aquatic studies with amphibians in the laboratory [10,22,24–31] and the field [12,14,23,32,33]. Because the effects of carbaryl have been well studied for amphibians, it may serve as a model chemical for understanding how insecticides in the carbamate family may affect amphibian populations and communities in nature. Carbaryl is relatively short-lived in the aquatic environment (<4 d in cattle tank pond experiments) [14] and does not bioaccumulate [6,34]. Most studies with carbaryl have focused on how a single exposure applied early in larval development at or around postapplication environmental levels (≤4.8 mg/L) [35,36] affects endpoints at metamorphosis (size, time, and survival to metamorphosis). This exposure regime, however, may not represent realistic environmental exposures because application of pesticides may occur repeatedly throughout the agricultural growing season. The objective of our study was to determine how multiple exposures versus single exposure at different times during development affected green frog survival to metamorphosis and tadpole survival, tadpole mass, and tadpole developmental stage (for those that did not metamorphose before winter) when reared in low- and high-density ponds.

MATERIALS AND METHODS

Experimental design

Three egg masses of the green frog (Rana clamitans) were collected at the Baskett Wildlife Area near Ashland (Boone County, MO, USA) on July 22, 2000. Eggs were hatched in the laboratory at 23 to 25°C and were held until tadpoles were free swimming (Gosner stage 25) [37]. We mixed tadpoles from all clutches before use in our experiment to homogenize genetic variation so that treatment effects and clutch differences would not be confounded.

We created aquatic communities in 36 polyethylene ponds (1.85 m in diameter; 1,480-L volume) by adding 1,000 L of water, 1 kg of leaf litter, and plankton from natural ponds (500 ml of plankton/pond at four different times) in mid-June. Zoo-plankton populations were established in the ponds by the time tadpoles were introduced. The ponds were located in a fenced field at the University of Missouri Research Park in Columbia (Boone County). Screen-mesh lids covered each pond to exclude incidental predators and anuran colonists. Use of outdoor, artificial ponds increases the environmental relevance and maximizes the benefits of laboratory and field experiments by maintaining relatively controlled environments while incorporating natural elements such as sunlight and shifts in temperature that would be present in a typical pond.

We manipulated experimentally two factors in a fully crossed design with three replicates: initial larval density, and the frequency and timing of exposure to carbaryl. Our selected densities (low [20 tadpoles] or high [60 tadpoles] per 1000 L) were within the range of tadpole densities in natural communities (14–4,238 per 1,000 L) [3,38]. Groups of 20 or 60 free-swimming tadpoles (Gosner stage 25) [37] were randomly assigned to ponds on July 29 (experimental day 0). Gosner stage 25 was selected because it was early in development and approximately 3 d after hatching, at which time the tadpoles were just beginning to feed.

We had six carbaryl dosing treatments to distinguish multiple effects from timing of exposure during larval development. Tadpoles were allowed a 12-d acclimation period, after which time we exposed tadpoles to zero to three doses of 3.5 mg/L carbaryl at 14-d intervals, i.e., at any time a dose was administered, the pond was exposed to 3.5 mg/L of carbaryl. A 14-d interval was selected based on recommended application rates for Sevin of every 7 to 10 d, or as needed. Ponds were exposed zero, one, two, or three times on days 12 (August 10), 26 (August 24), or 40 (September 7). Ponds that were exposed to carbaryl one time were dosed on day 12, 26, or 40 (in text referred to as early, mid, or late development); ponds that were dosed twice were exposed on days 12 and 26; and ponds that were dosed three times were exposed on days 12, 26, and 40. In this way, chemical treatments were applied on three different dates, and all ponds that were exposed multiple times were exposed on day 12. Developmental stage at the time of dosing was not determined to minimize disturbance of tadpoles in the pond community. We added carbaryl as liquid Sevin (21.3% carbaryl) at a nominal concentration of 3.5 mg/L (16.43 g Sevin added to 1,000 L water), which is below expected postapplication levels in wetlands receiving direct overspray (<4.8 mg/L) [35,36] and at levels known to alter behavior in many larval anurans [26,26]. Bridges (C. Bridges, unpublished data) found no difference between the effects of the commercial formulation of carbaryl (Sevin) and technical-grade carbaryl in laboratory mortality studies with amphibians; we selected the commercial formulation of carbaryl because it represents a realistic exposure scenario, whereas use of technical-grade carbaryl (which must be diluted in a carrier such as acetone) would not. We mixed carbaryl with 5 L of pond water and poured the mixture evenly across the pond surface with a watering can between 9:00 and 10:00 AM central standard time on each dosing date; we added 5 L of uncontaminated pond water to control ponds to mimic the disturbance of chemical application. We did not stir ponds to minimize the potential of an algal bloom and because direct application in the environment would not involve vigorous mixing.

Carbaryl exposure concentrations were sampled at 1, 24, 48, and 96 h following the first application of carbaryl. A 1-L sample was taken from three ponds exposed to carbaryl, from which 20 ml was removed, refrigerated, and sent to Mississippi
State Chemical Laboratory (Mississippi State University, Mississippi State, MS, USA) for high-performance liquid chromatography analysis. These water samples confirmed dosing exposure and indicated carbaryl had a half-life of approximately 4 h in our ponds (1 h, 2.1 mg/L; 24 h, 0.19 mg/L; 48 h, 0.12 mg/L; 96 h, 0.033 mg/L). For this reason, it is unlikely that any significant levels of carbaryl remained when ponds were dosed for a second or third time.

We measured each experimental unit (i.e., pond) for pH, temperature, and dissolved oxygen on each of the dates given above at 9:00 AM central standard time. Composite water samples of 6 L were taken to estimate chlorophyll levels on four dates (before chemical application on August 10, and after the first application on August 16, August 30, and September 20, 2000) from each experimental unit. A subsample of 100 ml was filtered and placed in 15 ml of neutralized 90% acetone in the dark at 5°C for 24 h prior to fluorometry [39]. The experiment was terminated on October 24, 2000 (day 87), when it appeared unlikely that any other individuals would reach metamorphosis before winter.

Response variables and statistical analyses

We searched ponds daily for metamorphs, defined by the emergence of at least one forelimb (stage 42) [37]. Metamorphs were removed from each pond and kept in the laboratory until tail resorption (usually ≤4 d), at which time they were towel-dried and weighed to the nearest milligram. At the end of our experiment, we drained ponds and thoroughly searched for any remaining tadpoles or metamorphs; all ponds contained tadpoles at that time. Each tadpole's developmental stage was determined and each was weighed to the nearest milligram.

Tadpole mass, developmental stage [37], and survival (number of tadpoles survived/stocking density-number of metamorphs collected), as well as the proportion reaching metamorphosis (number of metamorphs/stocking density) were used to measure the response of green frogs to density, dosing treatment, and their interaction. Analyses for treatment effects and their interactions on these responses were performed using analyses of variance. Mass and developmental stage were used as covariates for one another and tadpole survival was used as a covariate for both because these covariates explained significant proportions of the variation. To normalize data and stabilize variances, we angularly transformed proportion data, log transformed mass, and used a ranking procedure on developmental stage before analyses. We tested five null hypotheses using a priori orthogonal contrasts: Controls were not significantly different from exposure to carbaryl one, two, or three times (allowing us to determine if multiple exposures were different from controls); controls were not significantly different from single exposures that occurred early, mid, or late in development (allowing us to determine if a single exposure was different from controls); exposure once early in development was not significantly different from exposure to carbaryl two or three times (allowing us to determine if a single dose early in development was different from multiple exposures); two exposures were not significantly different from a single exposure middevelopment (allowing us to determine if a single exposure middevelopment was different from two exposures); and three exposures were not significantly different from a single exposure late in development (allowing us to determine if a single exposure late in development was different from three exposures). These contrasts allowed us to distinguish how timing of exposure differed from multiple exposures.

Water chlorophyll, pH, dissolved oxygen, and temperature were analyzed with a repeated-measures analysis of variance to determine differences of dosing treatment, density, and their interaction over time. Temperature, dissolved oxygen, and chlorophyll levels were log transformed before analysis.

RESULTS

In this study, we evaluated the effects of carbaryl and density on four critical amphibian endpoints, those being proportion metamorphosed and tadpole survival, mass, and developmental stage.

Proportion metamorphosed

Density, dosing treatment, and a density-by-dosing interaction significantly influenced the number of green frog tadpoles that reached metamorphosis (i.e., Gosner stage 42; Table 1; Fig. 1A). No frogs reached metamorphosis in control ponds and all (except one frog) that reached metamorphosis were from low-density ponds. The significant density-by-dosing interaction demonstrated that high-density conditions did not favor green frogs reaching metamorphosis, while low-density ponds exposed to carbaryl did; additionally, only those low-density ponds exposed to carbaryl early in development produced metamorphs (including all ponds exposed one, two, or three times, because they all had an early exposure). Low-density ponds that were dosed once early in development produced more metamorphs than ponds that were exposed to carbaryl once mid and late development at either density; this result indicates that the timing of the carbaryl exposure can affect whether or not a tadpole will reach metamorphosis. Low-density ponds that were exposed two and three times to carbaryl produced more and less metamorphs, respectively, than ponds dosed once early in development. However, according to a priori contrasts on the effects of dosing treatments, exposure to carbaryl once early in development produces the same effect (statistically) as two or three doses of carbaryl. The a priori contrasts also indicated that multiple exposures resulted in significantly more metamorphs than control ponds, while ponds exposed once during development did not produce significantly more metamorphs than controls (Table 1). This indicated that multiple exposures may be more environmentally important than single exposures. Dosing twice versus once middevelopment resulted in significantly more metamorphs (Table 1) according to a priori contrasts on the effects of dosing treatments, indicating that the cumulative exposure rather than exposure middevelopment alone resulted in this difference. Nineteen metamorphs were collected from four treatments (mean ± 1 standard error), but there were not enough individuals from the treatments to conduct statistical analyses: low-density ponds that were dosed once early (mass at metamorphosis, 1.007 ± 0.149 g; time to metamorphosis, 51.3 ± 2.3 d), twice (mass at metamorphosis, 1.704 ± 0.405 g; time to metamorphosis, 58.9 ± 1.6 d), or three times (mass at metamorphosis, 1.191 ± 0.133 g; time to metamorphosis, 68.8 ± 3.9 d); and a high-density pond that was dosed twice (mass at metamorphosis, 0.729 g; time to metamorphosis, 56 d).

Tadpole survival

The tadpole density, dosing treatment, and their interaction did not have a significant effect on tadpole survival (Table 1).
Although dosing treatment did not significantly affect tadpole survival, a priori contrasts of the effect of dosing treatments on green frogs were significant or near significant. Contrast analyses indicated that ponds with multiple exposures showed a trend of having greater tadpole survival than controls, while single-exposure treatments did not (Table 1; Fig. 1B). Additionally, the effect of three exposures to carbaryl resulted in significantly greater survival than those exposed once late in development (Fig. 1B). The most exposure to carbaryl (three exposures) resulted in relatively high survival and indicated that timing of a dose late in development alone is quite different from the cumulative effect of three exposures to carbaryl.

Developmental stage

Tadpole development was significantly reduced by greater density and significantly affected by dosing treatment but not by the interaction of density and dosing treatments (Table 1). Generally, green frogs exposed to carbaryl showed a trend of greater development (Gosner stage) at the end of the study than those reared in control ponds (Table 1; Fig. 2). Multiple exposures to carbaryl resulted in significantly enhanced development over controls, according to a priori contrasts on the effects of dosing treatment on green frogs. In contrast, controls were not statistically distinguishable from those exposed once during development, suggesting that multiple exposures can have a greater effect on green frogs than single exposures. Exposure to carbaryl three times resulted in significantly more developed tadpoles compared with those exposed once late in development, according to a priori contrasts on the effects of dosing treatment on green frogs. Because survival was also reduced in those ponds dosed once late in development (and because survival was used as a covariate), this suggests carbaryl caused mortality to those at more developed stages when
Timing of exposure versus multiple exposures to carbaryl

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Fig. 1. (A) Proportion metamorphosed in high- and low-density ponds across dosing treatments and (B) tadpole survival across dosing treatments. The numbers 0, 1, 2, or 3 represent the number of times ponds were exposed to carbaryl, and E, M, and L indicate if exposure occurred early, mid, or late during development, respectively. See Table 1 for significant contrasts.

Fig. 2. Gosner developmental stage of green frogs across dosing treatments. The numbers 0, 1, 2, or 3 represent the number of times ponds were exposed to carbaryl, and E, M, and L indicate if exposure occurred early, mid, or late during development, respectively. See Table 1 for significant contrasts.

Fig. 3. Mass of tadpoles at the end of the study across dosing treatments. The numbers 0, 1, 2, or 3 represents the number of times ponds were exposed to carbaryl, and E, M, and L indicate if exposure occurred early, mid, or late during development, respectively. See Table 1 for significant contrasts.

Fig. 4. Chlorophyll concentration in ponds over time.

The addition of carbaryl was a singular event. However, Gosner developmental stage ranged from an average 30 to 33, so differences among treatments were not biologically large.

Tadpole mass

Mass of green frog tadpoles was significantly reduced by increased density and moderately affected by dosing treatment but not affected by the interaction of density and dosing treatments (Table 1). Multiple doses of carbaryl had a greater effect on tadpole mass than a single dose of carbaryl during development (Table 1; Fig. 3). Additionally, the mass of frogs in ponds dosed three times was significantly less than those exposed once late in development, according to a priori contrasts on the effects of dosing treatment on green frogs.

Chlorophyll and water measurements

Chlorophyll levels were significantly affected by time (Fig. 4; Wilks $\lambda = 0.4306$, $F_{3,18} = 7.93$, $p = 0.0014$) but not by density (Wilks $\lambda = 0.7402$, $F_{3,18} = 2.11$, $p = 0.1351$), dosing treatment (Wilks $\lambda = 0.5315$, $F_{15,50} = 0.86$, $p = 0.6108$), or the interaction of density and dosing treatments (Wilks $\lambda = 0.7242$, $F_{15,50} = 0.41$, $p = 0.9681$) over time. Water temperature was significantly affected by time (Table 2; Wilks $\lambda = 0.0004$, $F_{2,19} = 23.683.7$, $p < 0.0001$) but not by density (Wilks $\lambda = 0.9561$, $F_{2,19} = 0.44$, $p = 0.6528$), dosing treatment (Wilks
Table 2. Summary of pH, dissolved oxygen ([DO]; μg/L), and temperatures (T; °C) in ponds on the three dosing dates. E, M, and L indicate, respectively, the time of exposure early (day 12), mid (day 26), or late (day 40) during tadpole development

<table>
<thead>
<tr>
<th>Dosing</th>
<th>Time</th>
<th>Date</th>
<th>pH</th>
<th>DO</th>
<th>T</th>
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<tbody>
<tr>
<td>E</td>
<td>Aug 10</td>
<td>8.4 ± 0.07</td>
<td>5.9 ± 0.2</td>
<td>28.0 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>Aug 24</td>
<td>8.6 ± 0.07</td>
<td>7.1 ± 0.07</td>
<td>24.9 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>Sep 6</td>
<td>8.7 ± 0.07</td>
<td>7.6 ± 0.2</td>
<td>20.2 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

λ = 0.6880, $F_{10,38} = 0.78$, $p = 0.6459$, or the interaction of density and dosing treatments (Wilks $\lambda = 0.5096$, $F_{10,38} = 1.52$, $p = 0.1691$) over time. Dissolved oxygen was significantly affected by time (Table 2: Wilks $\lambda = 0.3865$, $F_{2,19} = 15.08$, $p < 0.0001$) but not density (Wilks $\lambda = 0.9714$, $F_{2,19} = 0.28$, $p = 0.7588$), dose treatment (Wilks $\lambda = 0.6472$, $F_{10,38} = 0.92$, $p = 0.5227$), or the interaction of density and dose treatments (Wilks $\lambda = 0.9100$, $F_{10,38} = 0.18$, $p = 0.9965$) over time. Pond pH was not affected by time (Table 2; Wilks $\lambda = 0.8102$, $F_{2,19} = 2.22$, $p = 0.1355$), or density (Wilks $\lambda = 0.9850$, $F_{2,19} = 0.14$, $p = 0.8661$), dose treatment (Wilks $\lambda = 0.7123$, $F_{10,38} = 0.70$, $p = 0.7161$), or the interaction of density and dose treatments (Wilks $\lambda = 0.8676$, $F_{10,38} = 0.28$, $p = 0.9820$) over time.

**DISCUSSION**

Distinguishing the effects of multiple exposures from the timing of a single exposure may have important management and conservation implications. Because many of the pesticides in use today have a shorter half-life than early synthetic pesticides, they are believed to be safer, although they are often applied more frequently. Understanding how the timing of the exposure or multiple exposures may affect nontarget species like amphibians is necessary to adequately weigh the risks. Our study was designed as an expansion of a study by Boone et al. [23], which suggested that the combined stress of competition (mediated through larval density) and multiple chemical exposures lead to precocious metamorphosis and small metamorph size. However, it was difficult to distinguish effects of timing of exposure from multiple applications in their study because these factors were confounded.

Our results suggest that the high rates of metamorphosis found by Boone et al. [23] in high-density ponds exposed to carbaryl three times was likely a result of multiple exposures rather than solely the timing of exposure. Multiple exposures resulted in significant or near significant effects on survival to metamorphosis, tadpole survival, tadpole developmental stage, and tadpole mass, while single exposure during development did not. However, it must also be noted that all multiple dosed treatments were exposed early in development and that a priori contrasts between exposure once early in development and multiple exposures was never significantly different; early exposure may be critical in effects that occur with subsequent exposures. Overall, however, single exposure to carbaryl affected the anuran population less than multiple exposures. Our data also indicate that single exposures at three different times during development differ from multiple exposures. Exposure to carbaryl three times versus once late in development was significantly different at all endpoints except for proportion of tadpoles that reached metamorphosis. Tadpole survival was significantly lower in ponds exposed to carbaryl once late in development, and those tadpoles not reaching metamorphosis were less developed at the end of the study than those exposed to carbaryl three times. This result suggested that more developed tadpoles were killed by a single exposure late in development; otherwise, we would expect developmental stages similar to controls or enhanced development as in other exposure treatments. Differences between multiple effects and timing of exposure suggest that both may elicit effects on tadpoles during development but that, overall, multiple effects are larger.

In our present study and in Boone et al. [23], we observed that only ponds exposed to carbaryl produced metamorphs, indicating that presence of carbaryl could stimulate metamorphosis. In several studies with carbaryl using green frogs (species that typically overwinter as tadpoles in this area), we have consistently found that only tadpoles exposed to carbaryl reached metamorphosis and that those in control ponds do not [14,23]; additionally, in a study with bullfrogs, while no tadpoles reached metamorphosis during the first season, the developmental stage of bullfrog tadpoles in control ponds was significantly lower than those in ponds treated with carbaryl (M. Boone, unpublished data). If a tadpole does not reach metamorphosis (as in control ponds), it has additional time for larval growth—a trait that is positively correlated with fitness. Stimulation of metamorphosis in ponds exposed to carbaryl may be mediated through the production of stress hormones, which have been found to initiate metamorphosis in stressful drying environments [40,41], or through changes in the food web that enable tadpoles to reach metamorphosis more quickly (e.g., changes in algal resources) [32]. Tadpoles that are able to reach metamorphosis swiftly and leave a contaminated environment may be making an adaptive decision that increases their likelihood of survival if the terrestrial environment is more favorable. However, because many individuals died during tail resorption in Boone et al. [23], precocious metamorphosis may not necessarily be an adaptive response. Tadpoles have been found to be more sensitive to environmental changes during late stages of metamorphosis in some cases [9,22], but not in others [42]. Although we did not see any mortality at or around metamorphosis, pond cloudiness and/or presence of leaf litter may have precluded such observations in some ponds. Additionally, the precise timing during development may determine the lethality of a contaminant stress [10].

In our present study, most individuals that reached metamorphosis were from low-density ponds, whereas most metamorphs in Boone et al. [23] were from high-density ponds. Our high-density ponds may have been more stressful environments than in Boone et al. [23], although the cause for differences is not clear (but could be due to differences in algal resources, temperatures, etc.). The trend of finding metamorphs solely in carbaryl-treated ponds, however, is consistent among studies. Additionally, environmental conditions in both studies affected the outcome of whether or not metamorphosis was reached, although which specific environmental conditions differed among years.

Boone et al. [23] suggested that, because individuals reaching metamorphosis were relatively small, they may be less likely to survive the winter. In our present study, individuals that reached metamorphosis had a mass of greater than 1.0 g (most were from low-density ponds exposed to carbaryl), whereas most metamorphs in Boone et al. [23] weighed less than 0.6 g (most metamorphs were collected in high-density ponds exposed to carbaryl three times). The difference in size
between metamorphs in these two studies is likely attributable to density effects. However, individuals that reached (and survived) metamorphosis in Boone et al. [23] were reared in terrestrial enclosures and roughly 38% of them survived until the following spring, which was comparable with overwinter survival of other species reared in the enclosure study (unpublished data). This suggests that those surviving to metamorphosis in the fall can successfully overwinter, but it is unknown how survival may be influenced by those tadpoles that overwinter.

Carbaryl in our present study and Boone et al. [23] stimulated larval development of tadpoles, although development did not increase with each subsequent exposure. In our study and Boone et al. [23], mass was not significantly affected by dosing treatment; however, tadpoles from ponds dosed once late were significantly larger than those from ponds dosed three times, even with differences in survival taken into account (survival was used as a covariate). It is surprising that exposure to a single dose of carbaryl late in development resulted in tadpoles attaining a greater mass than tadpoles exposed to carbaryl three times; however, tadpoles in this treatment did have 47 d following carbaryl exposure before the experiment was terminated, and there could have been differences in food levels that our coarse measurements of pond chlorophyll did not reveal.

Our results suggest that multiple exposures during anuran development may affect the population more than a single exposure. However, single exposures at different times in development also can exert an effect different than what is found from multiple exposures. This work, in addition to Boone et al. [23], suggest that even one exposure to carbaryl, which had a half-life of only 4 h in our study, can stimulate development and may increase the rate of metamorphosis. How these effects may ultimately affect the population and community is not entirely clear. Rapport et al. [43] purports that any anthropogenic factors that have positive or negative effects should be viewed unfavorably because it is altering the trajectory of the community. Carbaryl consistently stimulates some level of metamorphosis in anurans with long larval periods, but how this effect may differ among species or how this effect may influence population dynamics is not clear. Future research could address how other amphibian species with similar (e.g., long larval periods) and different (e.g., short larval periods, different sensitivities to sublethal exposures of carbaryl) larval characteristics may respond to multiple exposures of a pesticide as well as how amphibians may be affected after overwintering and their subsequent performance in the terrestrial environment to gain a better understanding of the effects of multiple exposures to a contaminant. In our present study, we were able to demonstrate that sublethal exposure to an insecticide having a half-life of 4 h could influence tadpole development and rates of metamorphosis and it suggests that studies to examine the mechanisms of these effects are warranted.

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