

EFFECTS OF AN INSECTICIDE ON AMPHIBIANS IN LARGE-SCALE EXPERIMENTAL PONDS

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Abstract. We examined the effects of the insecticide carbaryl on larval amphibian communities in large-scale experimental ponds. Tadpoles of two anurans, Woodhouse's toad (*Bufo woodhousii*) and southern leopard frog (*Rana sphenocephala*), were reared in ponds (800 m³ volume) to determine the effects of tadpole density and carbaryl exposure on mass at metamorphosis and on time and survival to metamorphosis. Exposure to carbaryl significantly affected toads at metamorphosis, but not leopard frogs. Carbaryl exposure nearly doubled toad survival compared to controls; this effect may be attributable to an indirect effect of carbaryl increasing algal food resources. The competitive environment (i.e., density) and carbaryl exposure significantly affected the trade-off between mass and time to metamorphosis for toads. Our study is the first to demonstrate that in pond communities where predation and competition may be strong, short-lived insecticides can significantly alter the community dynamics of amphibians.

Key words: amphibian decline; anuran; *Bufo woodhousii*; carbaryl; food web; insecticide; larval period; *Rana sphenocephala*; southern leopard frog; Woodhouse's toad.

INTRODUCTION

The importance of natural biotic and abiotic factors on species persistence, diversity, and community interactions has been a pivotal focus for ecological researchers. Incorporating anthropogenic stressors into this framework is instrumental in explaining current trends related to persistence and diversity of species. Recent toxicological studies have shown that environmentally relevant levels of pesticides can affect population dynamics through altered abundance from differential tolerance and by reduced growth, which may affect fitness (Fairchild et al. 1994, Diana et al. 2000, Semlitsch et al. 2000, Boone and James 2003). Laboratory toxicity tests are designed to assess the impact of single pesticides under simplified environmental conditions to determine the direct effects of contaminants on individuals or populations. Yet, because natural environments include a large range of factors that are typically excluded in laboratory studies, pesticide levels that are safe in the laboratory or in mesocosms, or that are trivial in one-factor studies, may be far more detrimental to wild populations.

The most extensive examination of the effects of a single pesticide on amphibians has focused on the insecticide carbaryl, a carbamate neurotoxin that has low toxicity for amphibians (Bridges 1997, Boone and Semlitsch 2001, 2002) at realistic environmental con-

centrations (≤ 4.8 mg/L; Norris et al. 1983). This short-lived chemical does not bioaccumulate (Cox 1993) and can alter survival, body mass, and time to metamorphosis for some amphibian species at relevant environmental concentrations in the laboratory and field (reviewed in Boone and Bridges 2003). Field studies indicate that carbaryl affects amphibian communities through changes in the food resource base (i.e., indirect effects) rather than direct effects on individual physiology (Mills and Semlitsch, *in press*). The indirect effect occurs largely by reducing or eliminating zooplankton populations, which are sensitive to insecticides (Mayer and Ellersieck 1986). A reduction in zooplankton can result in subsequent algal blooms that can increase survival and mass at metamorphosis for herbivorous anurans (Boone and Semlitsch 2002, Mills and Semlitsch, *in press*) when detrimental direct effects are small compared to indirect benefits.

Studies using cattle tanks as artificial ponds have been successful in elucidating the role of natural factors on amphibian community processes (e.g., Morin 1983, Wilbur 1987). Although cattle tank studies can incorporate important factors known to influence natural communities, they still represent simplified, and often favorable, environments. For instance, cattle tanks typically yield survival rates $>50\%$ (e.g., Alford and Wilbur 1985, Wilbur and Fauth 1990) compared to $<5\%$ in natural ponds (Semlitsch 1987, Berven 1990, Semlitsch et al. 1996). Stresses in natural ponds could be so great that subtle chemical effects demonstrated in mesocosm studies may be obscured by strong competition or predation. For these reasons, it is necessary to understand if contaminants can have an impact on non-target wildlife under more natural field conditions.

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Additionally, because pesticides are believed to contribute to amphibian population declines (Wake 1991, Corn 1994) and have been correlated with some declines (Davidson et al. 2001), understanding their effect on amphibians is critical in establishing needed causal links. The objective of our study was to test the effects of carbaryl on two anuran species, Woodhouse's toad (*Bufo woodhousii*) and southern leopard frog (*Rana sphenoccephala*), experiencing different levels of competition in wetland mesocosms to determine the ecological consequences of pesticide exposure in experimental pond mesocosms.

METHODS

Species collection and experimental design

We collected ~20 egg masses of Woodhouse's toads and 28 egg masses of southern leopard frogs near Jefferson City (Callaway County), Missouri, USA, in flooded agricultural fields on 22 April 1999. This population of southern leopard frogs is known to hybridize with the plains leopard frogs, *Rana blairi*. Electrophoretic analysis of two loci indicated that 39% of the leopard frog egg mass used in our study contained some hybrid genotypes; however, a previous study indicated no significant differences between the response of the plains or southern leopard frogs to carbaryl (Boone and Semlitsch 2002). Eggs hatched and were held in the laboratory at 23°–25°C.

We used six experimental ponds at the USGS Columbia Environmental Research Center in Columbia (Boone County), Missouri. The ponds (8 × 10 m) had an average volume of 167 000 L and were ~1 m deep. These ponds were pumped dry in March 1999 to eliminate any overwintering tadpoles, fish, or aquatic predators that may have been present. We refilled ponds over 19–23 April with well-water (pH 7.8, hardness 286 mg/L as CaCO₃, alkalinity 258 mg/L as CaCO₃). Local amphibians (namely cricket frogs, *Acris crepitans*) and insect predators were free to colonize these ponds once they were refilled. During March and April, we enclosed each pond with a burlap drift fence (1 m height) that was buried 8 cm in the ground. We divided ponds into four quadrants using a polyethylene liner that prevented pesticide movement among quadrants and served as a barrier to tadpole dispersal. We buried the liner 6 cm in the pond bottom and weighted the bottom edge of the plastic with cement blocks for additional security. Four pitfall traps (8-L buckets) were placed in each quadrant along the inside edge of the drift fence to capture metamorphs as they left the pond.

We manipulated two factors (carbaryl and density) in a fully crossed design with each pond containing all four treatments and with each pond serving as a block, yielding six replicates and 24 experimental units; treatments within a block were assigned randomly. We manipulated carbaryl concentration (absent or 5.0 mg/L) and initial larval density (low [818 tadpoles total; ~20

tadpoles/1000 L] or high [2454 tadpoles total; ~60 tadpoles/1000 L]) at realistic field densities (14–4238 tadpoles/1000 L; e.g., Morin 1983, Petranka 1989). We mixed multiple egg masses within each species before adding them to the ponds to minimize biases due to clutch differences. We assigned groups of tadpoles randomly when they were free-swimming (Gosner stage 25; Gosner 1960). We reached initial larval densities by adding 198 (low density) or 594 (high density) Woodhouse's toads on 10 May, and 620 (low density) or 1860 (high density) southern leopard frogs on 11–12 May.

We added carbaryl (liquid Sevin, 21.3% carbaryl, Ortho, Columbus, Ohio, USA) on 18 May (day 0) between 16:00–18:00 Central Standard Time (CST). Carbaryl was diluted in ~20 L of pond water and sprayed evenly across the pond surface with a backpack chemical sprayer to simulate direct surface application. We chose our chemical level based on post-application, expected environmental concentrations of ~5.0 mg/L (Norris et al. 1983), which might result from direct overspray (e.g., for mosquito control). At the time of exposure, water temperature was 24.1° ± 0.1°C, pond pH was 8.0 ± 0.09, and dissolved oxygen was 9.4 ± 0.6 mg/L.

Water quality

We took 3-L composite water samples from three ponds exposed to 5.0 mg/L carbaryl at 1, 12, 48, and 96 h following carbaryl application that were analyzed by high-pressure liquid chromatography (HPLC) at Mississippi State Chemical Laboratory (Mississippi State, Mississippi, USA). The amount of carbaryl recovered at 1 h (8.05 mg/L, at this time it was unlikely that carbaryl would have been evenly distributed throughout the water), 12 h (2.92 mg/L), 48 h (1.38 mg/L), and 96 h (1.01 mg/L) indicated that carbaryl had a half-life of 22 h (the point at which the carbaryl concentration reached 2.5 mg/L, as interpolated from a plot). Composite water samples of 6 L were taken to estimate chlorophyll levels and zooplankton populations on five dates (before chemical application on 17 May and after chemical application on 20 May, 26 May, 9 June, and 13 July) from each quadrant. Chlorophyll concentration was determined from a 100-mL subsample that was filtered and placed in 15 mL of neutralized 90% acetone at 5°C for 24 h; chlorophyll concentration was determined with fluorometry (Greenberg et al. 1992). A subsample of 1.6 L was filtered from each quadrant for zooplankton. Zooplankton were rinsed into a vial with 80% ethanol and were later identified to the level of cladoceran, copepod, or ostracod. We measured each quadrant for pH, temperature, and dissolved oxygen on each sampling date at 09:00 CST.

Response variables and statistical analyses

We searched pitfall traps and ponds daily for metamorphs (Gosner stage 42; Gosner 1960). We removed

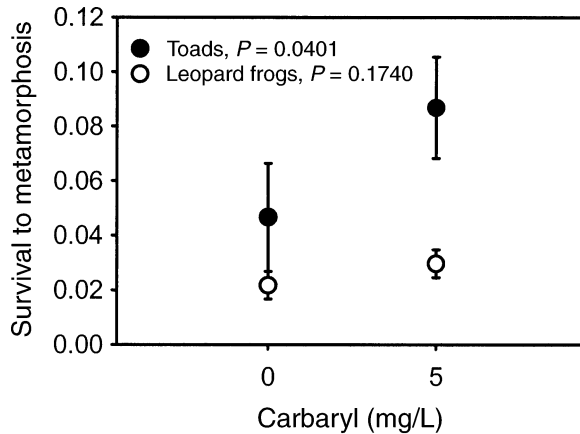


FIG. 1. Average survival to metamorphosis for Woodhouse's toads (*Bufo woodhousii*) and southern leopard frogs (*Rana sphenoccephala*) reared in control and carbaryl-treated environments. Error bars indicate ± 1 SE.

metamorphs from the traps or ponds, and weighed them to the nearest milligram on the date of capture. On 16 September 1999 (day 121), we ended the experiment because few metamorphs were found on daily checks and many ponds were dry.

Mass, time, and percent survival to metamorphosis were used to measure the response of each anuran species to density and carbaryl treatments. We used a multivariate analysis of variance (MANOVA) to test for the effects of pond block, density, carbaryl exposure, and the density by carbaryl interaction on mass and days to metamorphosis for each species, followed by univariate analyses of variance (ANOVA). Survival to metamorphosis was analyzed separately using an ANOVA because there were no survivors in one replicate, and in a MANOVA this replicate would be eliminated from the analysis due to missing values for mass and time; therefore, to include all available data, we analyzed survival separately. We normalized the data prior to analyses using angular (for survival to metamorphosis) or log (for mass and days to metamorphosis) transformations. Alpha was set at 0.1 a priori because high variability was expected to exist among pond blocks, which could obscure subtle chemical effects. One low-density control treatment was eliminated in analyses for Woodhouse's toads because local toads had apparently laid eggs in this quadrant; tadpoles were visible at the time we stocked tadpoles in this quadrant and more toads were captured here than we originally stocked. Chlorophyll, zooplankton, and pond water measures (pH, temperature, and dissolved oxygen) were analyzed with a repeated measure ANOVA to determine the main effects and interactions of time, pond block, carbaryl, and density. Chlorophyll and zooplankton counts were log-transformed.

RESULTS

Survival of Woodhouse's toads to metamorphosis was 1.8 times greater when exposed to carbaryl than

TABLE 1. Summary of univariate ANOVAs for survival, mass, and time to metamorphosis for Woodhouse's toads.

Response and source of variation	df	MS	F	P
Survival				
Pond block	5	0.0411	4.75	0.0096
Density	1	0.0505	5.83	0.0301
Chemical	1	0.0401	5.12	0.0401
Density \times chemical	1	0.0006	0.07	0.8005
Error	14	0.0087		
Mass				
Pond block	5	0.1116	2.33	0.1019
Density	1	0.0001	0.00	0.9689
Chemical	1	0.0089	0.19	0.6738
Density \times chemical	1	0.2388	4.98	0.0438
Error	13	0.0479		
Day				
Pond block	5	0.08517	5.11	0.0082
Density	1	0.0038	0.23	0.6388
Chemical	1	0.0131	0.79	0.3904
Density \times chemical	1	0.0632	3.79	0.0734
Error	13	0.0167		

in controls (Fig. 1, Table 1). The multivariate response of mass and time to metamorphosis was only significantly affected by pond block (Wilks' lambda = 0.2088, $F_{10,24} = 2.85$, $P = 0.0172$). However, univariate analysis indicated that mass and time to metamorphosis of toads was significantly affected by the carbaryl by density interaction (Fig. 2, Table 1), and this interaction was consistent in most pond blocks. With carbaryl exposure, toad tadpoles from low-density treatments consistently reached larger sizes at metamorphosis than those in high-density treatments; this relationship was reversed in chemical controls. Length of the larval period was essentially the same in low-density treatments regardless of carbaryl exposure, whereas at high-density conditions tadpoles had shorter larval periods in carbaryl treatments. Frequently, there is a trade-off be-

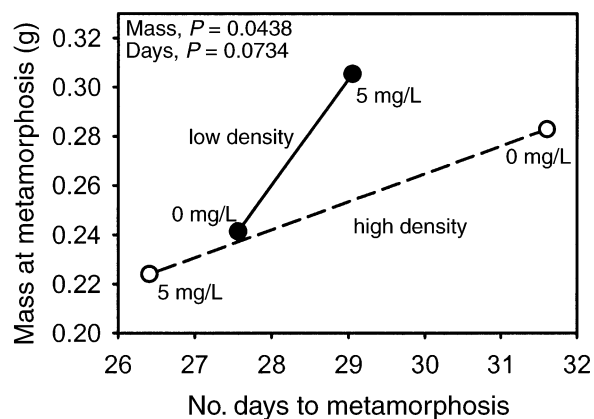


FIG. 2. The trade-off between mass at metamorphosis and days to metamorphosis of the Woodhouse's toads (*Bufo woodhousii*) reared in low- and high-density ponds at 0 and 5 mg/L carbaryl.

tween mass and time to metamorphosis, with individuals reaching a larger mass with a longer larval period. The trade-off between these factors was greatest in high-density treatments, with those in controls having longer larval periods and larger mass at metamorphosis (Fig. 2). In low-density treatments, however, a greater mass was obtained in carbaryl treatments with only a slightly longer larval period compared to controls. Mass and time to metamorphosis were significantly correlated ($r = 0.7269$, $P \leq 0.0001$), with smaller masses associated with shorter larval periods. Because of the correlation between these variables, multivariate analysis does not show an effect of the interaction of carbaryl and density that is apparent in the univariate analyses; size at metamorphosis can be explained by the length of the larval period in the MANOVA, but the interaction of the treatments appears to be driving this trade-off, and is, therefore, worth consideration. Neither carbaryl nor the interaction of carbaryl with density appeared to affect mass or time to metamorphosis or survival for the leopard frogs.

High-density conditions had a significant, negative effect on survival to metamorphosis for both toads and leopard frogs (toads, low density = 0.096 ± 0.020 [mean ± 1 SE], high density = 0.038 ± 0.019 [Table 1]; southern leopard frogs [$F_{1,15} = 20.19$, $P = 0.0004$], low density = 0.037 ± 0.005 , high density = 0.015 ± 0.005). Pond block significantly affected survival and the multivariate response of mass and days to metamorphosis for both toads (multivariate response [mass and days to metamorphosis], Wilks' lambda = 0.3623, $F_{10,28} = 1.85$, $P = 0.0969$; Table 1) and leopard frogs (survival, $F_{5,15} = 11.55$, $P \leq 0.0001$; multivariate response [mass and days to metamorphosis], Wilks' lambda = 0.2088, $F_{10,24} = 2.85$, $P = 0.0172$).

Water quality

Time and pond block had a significant effect on pH (time, $F_{4,12} = 97.02$, $P \leq 0.0001$; block, $F_{20,41} = 4.95$, $P \leq 0.0001$), temperature (time, $F_{4,12} = 1561.36$, $P \leq 0.0001$; block, $F_{20,41} = 2.93$, $P = 0.0018$), and dissolved oxygen (time, $F_{4,12} = 49.63$, $P \leq 0.0001$; block, $F_{20,41} = 5.01$, $P \leq 0.0001$), but treatments or their interactions did not. Cladocerans were negatively affected by time ($F_{4,11} = 46.91$, $P < 0.0001$), and significantly affected by pond block ($F_{20,37} = 3.07$, $P = 0.0015$) and a carbaryl by time interaction ($F_{4,11} = 3.31$, $P = 0.0521$). Copepods were negatively affected by time ($F_{4,11} = 3.85$, $P = 0.0023$), and significantly affected by an interaction of time and carbaryl ($F_{4,11} = 3.85$, $P = 0.0341$). Ostracods changed significantly over time ($F_{4,11} = 72.95$, $P < 0.0001$) and were significantly affected by pond block ($F_{20,37} = 3.01$, $P = 0.0018$). In ponds exposed to carbaryl, the number of cladocerans sampled was significantly lower after carbaryl application on 20 May ($F_{1,14} = 8.54$, $P = 0.0111$), 26 May ($F_{1,14} = 8.61$, $P = 0.0109$), and 9 June ($F_{1,14} = 9.73$, $P = 0.0075$), but was equal to controls by the final

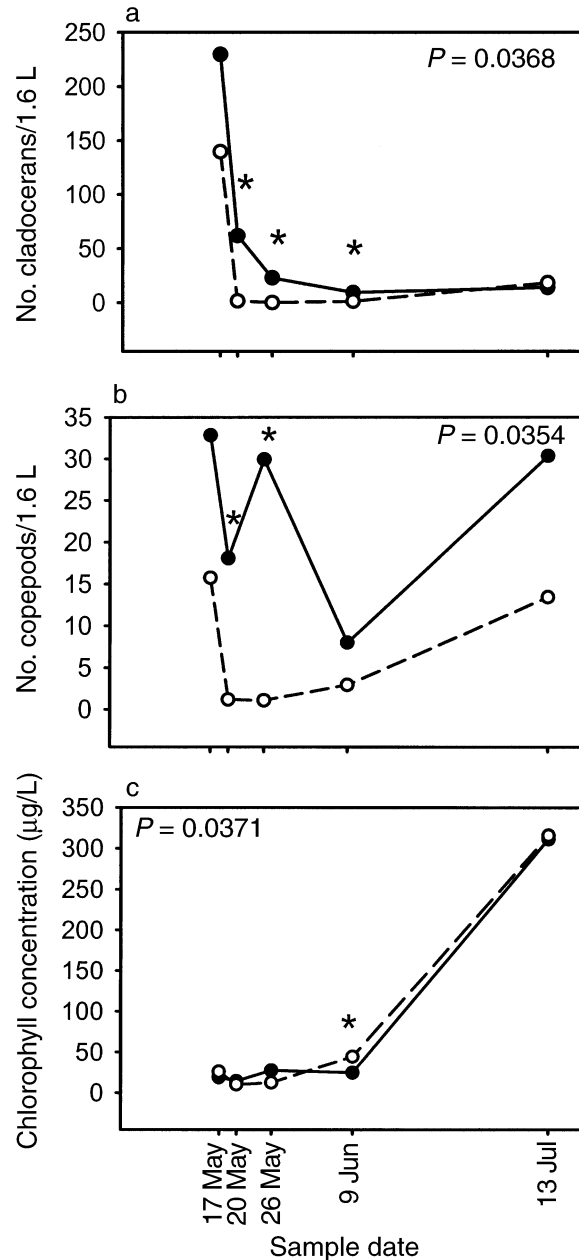


FIG. 3. The number of (a) cladocerans and (b) copepods collected from carbaryl treatments over time, and (c) the concentration of chlorophyll ($\mu\text{g/L}$) from a 100-mL water sample from carbaryl treatments over time. Solid lines and circles are the 0 mg/L carbaryl treatments, and dotted lines with open circles are the 5 mg/L carbaryl treatments. Asterisks (*) indicate significant differences ($P < 0.05$) according to univariate analyses of variance.

sample (Fig. 3a). Copepods were also reduced after carbaryl exposure on 20 May ($F_{1,14} = 54.98$, $P \leq 0.0001$), 26 May ($F_{1,14} = 20.21$, $P = 0.0005$), and 9 June ($F_{1,14} = 7.32$, $P = 0.0171$), but were similar to controls at the final sample (Fig. 3b). The standing crop of chlorophyll showed significant increases over time

($F_{4,11} = 70.38$, $P \leq 0.0001$), varied with pond block over time ($F_{20,37} = 1.82$, $P = 0.0566$), and varied with carbaryl exposure over time ($F_{4,11} = 3.74$, $P = 0.0371$). Carbaryl exposure increased the amount of chlorophyll measured on 9 June ($F_{1,14} = 5.07$, $P = 0.0409$), but chlorophyll was the same in exposed ponds as control ponds by the end of the study (Fig. 3c). There were no significant differences in chlorophyll concentration over time due density ($F_{4,11} = 0.66$, $P = 0.6331$), or the interaction of carbaryl and density ($F_{4,11} = 0.29$, $P = 0.8782$).

DISCUSSION

Our results indicate that even a short-lived contaminant may alter population dynamics under field conditions. In our study, carbaryl had a half-life of 22 hours, yet endpoints at metamorphosis were affected 30 or more days following exposure. The conditions experienced in these experimental ponds are very similar to natural environmental conditions of temporary wetlands: The ponds dried over the course of the season, insect and vertebrate predators were present, and additional competitors bred in these ponds, thereby increasing total larval densities. Approximately 3% of the tadpoles initially stocked reached metamorphosis, yielding survival rates similar to those in natural ponds (Semlitsch 1987, Berven 1990). Though the magnitude of a chemical effect may be small relative to factors such as competition or predation, these natural stresses did not mask the effect of carbaryl, and this indicates that carbaryl was a significant factor in the community dynamics of our study.

The results reported here are similar to findings in previous experiments in cattle tanks (e.g., Boone and Semlitsch 2002, Boone and James 2003), suggesting that cattle tanks are useful in testing the effects of a contaminant on a community. As previously demonstrated, southern leopard frogs were not affected at metamorphosis from carbaryl exposure (Boone and Semlitsch 2002). We have hypothesized that leopard frogs may be less affected by carbaryl exposure than other species because their long larval period allows them to overcome any short-term negative effects, or because these individuals were resistant to contaminants due to past exposure in agricultural fields surrounding the population source (as suggested by Bridges and Semlitsch 2001). The realistic conditions of our ponds did not appear to make this species more or less sensitive to carbaryl.

Exposure to carbaryl early in larval development positively influenced toad survival in our study, similar to Boone and Semlitsch (2002). Even short-term exposure may be important for species with rapid growth and short larval periods. Because other studies have shown that exposure to carbaryl can lead to reduced (Boone and Semlitsch 2001) or increased (Boone and Semlitsch 2002) survival of toads, these results suggest that the direction of the effect may be difficult to predict

for anurans. Whether individuals are affected positively or negatively may be related to the susceptibility of individuals to the direct effects of carbaryl (Bridges and Semlitsch 2000) or to the magnitude of indirect effects on the food web (Mills and Semlitsch, *in press*). However, because both positive and negative effects alter the community, both outcomes are signs of disruption of ecosystem function and food web dynamics (Rapport et al. 1985).

There are several ecologically relevant mechanisms to explain positive effects of carbaryl exposure. First, carbaryl levels used in our study can induce mortality for many aquatic predators including insects and crayfish that are more sensitive to insecticides (Mayer and Ellersieck 1986), which may have reduced tadpole mortality for a short period of time. If reduced predation was the cause for increased survival, we might expect leopard frogs to also benefit; slightly more leopard frogs did emerge from carbaryl treatments at larger sizes, although these differences were minor. However, leopard frogs spent an average of 52 days more in the aquatic environment than toads. Relief from predation may be temporary and may not result in any advantage to species with longer larval periods.

A second mechanism that may have increased toad survival is related to an indirect effect on the food supply. Zooplankton and tadpoles can compete for similar algal resources. Because carbaryl effectively reduces or eliminates zooplankton populations (Hanazato and Yasuno 1987, Havens 1995), more food resources are available for anuran tadpoles (Havens 1995) and the risk of dying due to starvation may be reduced, especially in highly competitive environments (Mills and Semlitsch, *in press*). Our plankton analyses indicate that carbaryl temporarily reduced some zooplankton classes, and increased algal resources. Elimination of zooplankton can encourage algal blooms (Havens 1995, Diana et al. 2000), which may have allowed toad tadpoles to experience greater survival. Our plankton data support this mechanism and could explain the increase in toad abundance. Species with longer larval periods, such as southern leopard frogs, may be less likely to benefit from short-term increases in food resources.

The environmental conditions of the pond (i.e., initial larval density) were important to the effect of the insecticide on the community, as previously observed in cattle tank studies (Boone and Semlitsch 2001, 2002, Boone et al. 2001). Under low-density conditions, carbaryl exposure increased mass at metamorphosis, whereas under high-density conditions carbaryl exposure reduced mass. A single stress of either density or carbaryl appeared to stimulate mass and time to metamorphosis, while the combination of these stresses reduced these measures. Interestingly, in control conditions, toads in low-density ponds had lower masses (16% less) than those in high-density ponds. Because toad tadpoles aggregate within a pond, which may stir

up food resources that can be filtered from the water (Beiswenger 1975), increased density can result in increases in mass (Wilbur 1977) and may explain this result. Additionally, changes in the predator environment from carbaryl exposure could also influence this interaction. Because carbaryl is lethal to invertebrate predators, reducing or eliminating this predator class (at least temporarily) could influence how amphibians make the trade-off between mass and time to metamorphosis. It is noteworthy that carbaryl exposure altered how animals in low- and high-density treatments made the trade-off. Whether the trade-off was related to a change in the predator environment, resource availability, or some other reason is unclear, although shifts in trade-offs between size and time to metamorphosis have also been exhibited by *Rana* tadpoles exposed to the fungicide triphenyltin (Fioramonti et al. 1997) and *Ambystoma tigrinum* exposed to atrazine (Larson et al. 1998).

In previous studies (Boone and Semlitsch 2001, 2002), anurans in high-density ponds "benefited" the most from carbaryl exposure compared to tadpoles in low-density ponds, presumably due to an increase in algal food resources as a result of a reduction in zooplankton. However, in our present study, carbaryl appeared to benefit individuals in low-density environments over those high-density environments. In more natural ponds, high-density conditions may be more severe because of increased predatory and competitive stresses compared with cattle tanks. Density levels in cattle tanks (e.g., Boone et al. 2001, Boone and Semlitsch 2001) and our experimental pond study were approximately the same (2 or 6 individuals per 10 L for low and high density, respectively), so other stresses likely account for the differences between our results and previous studies. This suggests that cattle tank experiments may underestimate contaminant effects in the natural environment.

In conclusion, our study demonstrates that a contaminant can influence population dynamics of amphibians in seminatural ponds, despite the strength of other stresses such as competition and predation. Our study also confirmed cattle tank or mesocosm studies are useful in understanding the role that contaminants may play in natural systems, although the conditions in these studies may be more favorable than natural ponds. We found that one species, the Woodhouse's toad, was influenced by carbaryl exposure. Ideally, a contaminant should have no observable effects on a population or community processes. The species used in our experiment also responded quite differently, and such variation in responses among species must be considered in conservation and management plans. Carbaryl has been viewed as a relatively safe chemical (Cox 1993) because it is short-lived in the environment. However, when a chemical with a half-life of <24 hours can influence a population, this suggests that even short-lived contaminants can alter populations

and that long-lived contaminants may be an even more serious threat. We contend that chemical stressors, in relation to physical and biological stressors, must continue to be evaluated to fully understand the extent and nature of local and global amphibian declines.

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