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APPLIED ISSUES

Interaction of an insecticide with larval density in pond-breeding salamanders (*Ambystoma*)

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SUMMARY

1. Amphibian populations residing in or near agricultural areas are often susceptible to pesticide contamination. Recent evidence suggests that the effects of pesticides on amphibians often exceed those estimated in laboratory toxicity tests because other environmental factors (e.g. predators, resource abundance) can influence pesticide toxicity.
2. To examine the effects of an insecticide (carbaryl) on two species of *Ambystoma* salamanders experiencing the natural stress of competition, we manipulated chemical concentration (control, 3.5 and 7.0 mg L^{-1}) and larval density (low and high). We determined the effect of treatments on snout-vent length (SVL), growth rate, lipid reserves, time to metamorphosis, per cent survival and per cent metamorphosis.
3. Carbaryl negatively affected all response variables of *Ambystoma maculatum* significantly, and significantly reduced survival and metamorphosis of *A. opacum*. Increased density significantly influenced SVL, lipid reserves, growth rate and metamorphosis of *A. maculatum*.
4. The effects of carbaryl and increased density on per cent metamorphosis were nearly additive, but were generally less than additive on other variables.
5. The negative effects of chemical contamination on salamanders were likely because of pesticide-induced reductions of food resources, as zooplankton abundance decreased by as much as 97% following carbaryl application.
6. Our study demonstrates the importance of the interactive effects that chemical contamination and natural environmental factors have on salamanders.

Keywords: *Ambystoma maculatum*, *Ambystoma opacum*, carbaryl, density, insecticide

Introduction

Some amphibian populations worldwide have declined in recent years (Barinaga, 1990; Blaustein & Wake, 1990; Wake, 1991; Houlahan *et al.*, 2000), however the causes of declines have been difficult to confirm. It is likely that several factors, including habitat loss or degradation, introduction of non-native species, disease and chemical contamination contribute to declines (Corn, 1994; Dodd, 1997; Carey, Cohen & Rollins-Smith, 1999). Of these, the overall importance of chemical contamination on amphibian populations is not well understood, but some evidence suggests that pesticides may contribute to amphibian declines on a regional scale (Davidson, Shaffer & Jennings, 2001).

Understanding how pesticides affect amphibian ecology is important for determining the impact of chemical contamination on amphibian populations. Although many studies have examined lethal-limits of pesticides on amphibians, environmental concentrations are frequently not high enough to induce

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direct amphibian mortality. Consequently, assessing the sublethal effects of pesticides may be more relevant. Current research suggests that exposure of amphibians to certain chemicals affects behaviour (Marian, Arul & Pandian, 1983; Bridges, 1997) and development (Bridges, 2000). Additionally, these sublethal effects can be exacerbated by environmental factors such as competition and predation (Boone & Semlitsch, 2001; Relyea & Mills, 2001). Understanding interactions between contaminants and environmental factors is ultimately important for drawing realistic conclusions about contaminant effects in complex ecological systems (Hopkins *et al*., 2002, 2004).

Carbaryl (1-naphthyl N-methyl carbamate) has become a model pesticide for testing the effects of carbamate and organophosphate insecticides on amphibians (Bridges & Semlitsch, 2000), and has proven useful in unveiling the complex ecological interactions that can be disrupted by pesticides (Boone & Semlitsch, 2001, 2002; Boone & James, 2003; Relyea, 2003). Carbaryl, much like other carbamate and organophosphate insecticides, is a short-lived insecticide that inhibits cholinesterases (Cox, 1993; Beyers & Meyers, 1996), thereby disrupting nervous system function. Carbaryl is applied to crops at a rate of approximately 3000 metric tons per year (United States Environmental Protection Agency, 1992) and is the active ingredient in many common garden insecticides (e.g. Sevin®). Carbaryl enters aquatic systems from direct application or agricultural runoff (Cox, 1993; Capel, Larson & Winterstein, 2001) and is one of the most common pesticides found in aquatic systems (Gilliom, 2001), thereby posing risks to aquatic and semiaquatic fauna.

Although carbaryl has low acute toxicity in some amphibians (e.g. anurans; Bridges, 1997; Boone & Semlitsch, 2001, 2002), the sublethal effects of carbaryl can be profound. For instance, in the laboratory, acute exposure to carbaryl reduces spontaneous activity and swimming performance (Bridges, 1997), increases the frequency of developmental abnormalities and reduces the size at which individuals metamorphose (Bridges, 2000). In contrast, exposure to carbaryl under more ecologically realistic conditions (i.e. in outdoor artificial ponds) often produces the opposite result. Carbaryl concentrations that are consistent with high field-application levels of 3.5–4.8 mg L^{-1} (Norris, Lorz & Gregory, 1983; Peterson *et al*., 1994) mainly affect anuran communities indirectly. Carbaryl rapidly reduces zooplankton abundance, which alters competitive interactions among periphyton, phytoplankton and anuran larvae (Mills & Semlitsch, 2004). Depending on community structure, exposure to carbaryl can thereby have indirect effects on anuran developmental rate, growth and duration of the larval period (Boone & Bridges, 1999; Bridges & Boone, 2003; Boone & Semlitsch, 2001, 2002; Mills & Semlitsch, 2004).

Despite the devastating effect that carbaryl has on invertebrate communities (Mayer & Ellersieck, 1986), few studies have examined the effects of pesticides on salamanders. Carnivorous salamander larvae that rely on zooplankton and other invertebrates as food may be negatively affected by carbaryl-induced reductions in food resources (Hanazato & Yasuno, 1987, 1990; Boone & James, 2003; Mills & Semlitsch, 2004). The few studies examining the effects of carbaryl on salamanders indicate it can decrease survival and growth of red spotted newts, *Notophthalmus viridescens* (Rafinesque) (Boone & Semlitsch, 2001; Mills & Semlitsch, 2004), spotted salamanders, *Ambystoma maculatum* (Shaw) and small-mouth salamanders, *Ambystoma texanum* (Matthes) (Boone & James, 2003).

The purpose of the current study was to assess the impact of carbaryl on two species of *Ambystoma* salamanders while in competition with one another in artificial ponds, and to investigate the influence of salamander density on responsiveness to the pesticide. Ecological variables such as density can impact per capita resources and thus the intensity of inter- and intra-specific competition (Wilbur & Collins, 1973; Wilbur, 1980). Semlitsch & Walls (1993) found that increasing larval density reduced growth rate, size and metamorphosis of two *Ambystoma* species. Similarly, Scott & Fore (1995) demonstrated that food availability affects growth, body condition and individual fitness of marbled salamanders. Although the interaction between carbaryl and density has been examined in anurans (Boone & Semlitsch, 2001, 2002; Mills & Semlitsch, 2004), no studies have examined how density influences the effects of carbaryl on salamanders. We hypothesised that by simultaneously increasing carbaryl and salamander density, the overall effect on salamanders would be more severe because of additive reductions in per capita food resources.

Methods

Species collection

Two species of salamanders, *A. maculatum* (spotted salamander) and *Ambystoma opacum* (Gravenhorst) (marbled salamander) were used in this study. The species can occur sympatrically and represent common constituents of natural communities in the eastern United States. *Ambystoma opacum* and *A. maculatum* have very different reproductive strategies. *Ambystoma opacum* lay their eggs terrestrially in the autumn and the embryos develop and hatch when inundated with water. In contrast, *A. maculatum* lay their eggs aquatically in winter after wetlands fill. However, in the area where we collected eggs, there can be considerable overlap in the timing of hatching of these two species depending on precipitation patterns and the hydroperiod of the wetland.

In November 2001 adult male and female *A. opacum* were collected from Ginger's Bay in Aiken County, S.C. Salamanders were placed in cattle tanks and allowed to breed and lay eggs terrestrially. On 25 February 2002, 12 *A. maculatum* egg masses were collected from wetlands along Risher Pond Road in Barnwell County, S.C. Egg masses were mixed (to homogenise genetic variation) and placed in a 20-L container of well water in the laboratory until they hatched on 8 March 2002. That same day, eggs from five *A. opacum* clutches were removed from their oviposition sites in cattle tanks. *Ambystoma opacum* clutches were mixed and put in water where the eggs hatched almost immediately. Hatching eggs of the two species on the same day ensured that larvae were similar in size and age at the start of the study.

Experimental design

Outdoor mesocosms ($n = 24$) were created in polyethylene cattle tanks (1.85 m diameter, 1480 L volume) located in a fenced field at the Savannah River Ecology Laboratory near Aiken, South Carolina. Mesocosms were filled with 1000 L of well water, 1.0 kg leaf litter and 14.6 kg soil/mud from a nearby Carolina bay, which likely contained zooplankton eggs. Previous studies examining the effect of carbaryl on amphibians have not introduced sediment into their mesocosms (Bridges, 1997; Boone & Semlitsch, 2001, 2002; Mills & Semlitsch, 2004). Because contaminants often bind to sediments and particulate matter (Capel *et al.*, 2001), the current technique may better mimic carbaryl dynamics under natural conditions. Screen mesh lids were used to cover each mesocosm to exclude colonising predators and competitors, and also to provide some shade. To ensure salamander larvae would have food resources, each mesocosm was inoculated with pond water containing concentrated zooplankton (500 mL of pond water per mesocosm) on three occasions during February and early March 2002.

Two factors, initial larval density and carbaryl concentration, were manipulated in a completely randomised design with four replicates per treatment combination. On 11 March 2002 (3 days after hatching) free-swimming larvae were randomly assigned to mesocosms. Six larvae of each species were added to create a low density of salamanders ($n = 12$) in half of the mesocosms, and 18 larvae of each species were added to create a high density ($n = 36$) in the remaining half. On 25 March 2002 carbaryl was added to the mesocosms by mixing liquid Sevin, which contains 22.5% carbaryl by weight, with 5 L of water from the mesocosm and pouring the mixture evenly across the surface with a watering can. To minimise disturbance, and because direct application in a natural environment would occur similarly, mesocosms were not stirred. Chemical concentrations were chosen based on previous exposure concentrations (Boone & Semlitsch, 2001, 2002): no-carbaryl control (0 mg L^{-1}), a low-carbaryl treatment (3.5 mg L^{-1} or 15.56 g liquid Sevin) and high-carbaryl treatment (7.0 mg L^{-1} or 31.11 g liquid Sevin). Although all concentrations used are within the range of concentrations possible in the field, the high-carbaryl treatment would be considered a worst-case scenario, and is known to be near the LC50 for some anuran species (Boone & Bridges, 1999; Bridges, 1999). No chemical analyses were conducted on the water, but studies suggest that carbaryl has a half-life of approximately 4 days in outdoor mesocosms (Boone & Semlitsch, 2001), and may be completely degraded in 16 days (Mills & Semlitsch, 2004).

Zooplankton were sampled twice during the study, once just before carbaryl application (25 March 2002) and again 2 weeks after carbaryl application (8 April 2002). A 1.0-L plastic tube (5 cm in diameter) was inserted into the water column at five predetermined locations in each mesocosm. The five samples were combined into a single sample and poured through an

80-μm zooplankton net. Using a dissecting scope, zooplankton were identified and counted to estimate invertebrate abundance.

Response variables and statistical analyses

Plastic minnow traps (three per mesocosm) were suspended in mesocosms and checked daily for metamorphs. Metamorphosis, defined as resorption of gills, began on 3 May 2002. Metamorphs were weighed (wet mass; mg) and measured (snout-vent length, SVL; mm), and the number of days to metamorphosis was recorded. Growth rate (mm day^{-1}) was then calculated by dividing SVL by the number of days to metamorphosis. A subsample of metamorphs from each mesocosm that produced metamorphs were then killed and frozen (–70 °C) for future lipid analysis. On 22–23 July 2002 (days 136–137) the experiment was terminated because few metamorphs had been captured in the previous 2 weeks, and because many ephemeral wetlands in our area are dry by this date. Mesocosms were drained and thoroughly searched for any remaining larvae.

Total non-polar lipid (NPL) content of salamanders was determined according to the methods of Hopkins *et al.* (2000). Frozen samples were lyophilised to a constant mass and dry samples were then extracted with petroleum ether for 4 h using a soxhlet apparatus. Extracted samples were then re-dried at 60 °C to a constant mass. Pre- and postextraction masses (nearest 0.01 mg) were acquired using a balance (Mettler Toledo AG285) housed in a dry box at 4% relative humidity. Total NPL for each individual was calculated by subtracting the dry postextraction sample mass from the dry pre-extraction sample mass.

Responses of *Ambystoma* larvae were compared using analysis of covariance (ANCOVA; SAS, 1999). Because only two salamanders metamorphosed from the 7.0 mg L^{-1} carbaryl treatment (see results) these data were not used in the statistical analysis of sublethal response variables. Also, cattle tanks from which no larvae successfully metamorphosed were excluded from the analysis of sublethal response variables. Because survival can affect life history traits including size at metamorphosis and larval period (Travis, 1983; Wilbur, 1997), it was included as the covariate in the analysis of the other dependent variables (e.g. Parris & Semlitsch, 1998). The dependent variables used were days to metamorphosis, size at metamorphosis, growth rate, per cent lipid, per cent survival and per cent metamorphosis. Because the two species and the response variables measured on individuals were not independent of each other, we applied a sequential Bonferroni adjustment to maintain an experiment-wide error rate of $\alpha = 0.05$. Mass and SVL produced similar results, so only SVL was used for the analysis of size at metamorphosis. The independent variables used in our models were initial larval density, carbaryl treatment and their interaction. All larvae that metamorphosed or that were still alive in the mesocosms at the termination of the experiment were considered survivors. Per cent survival and per cent metamorphosis were calculated by dividing the number of survivors and metamorphs by the number of larvae initially added to each mesocosm. To normalise the data, days to metamorphosis, SVL and growth rate were $\log_{10}$ transformed, and per cent lipid, survival and metamorphosis were arcsine square-root transformed.

Zooplankton abundance, expressed as individuals per litre, was $\log_{10}$ transformed and compared among treatments using repeated measures analysis of variance (ANOVA). The independent variables in the model were carbaryl concentration and initial larval density. The repeated statement in the model was time (i.e. before and after carbaryl treatment).

Results

Effects on the salamanders

Density and carbaryl significantly influenced growth and body condition of *A. maculatum* (Table 1), but not *A. opacum* during the study (Table 2). *Ambystoma opacum* metamorphs were generally larger (SVL) than *A. maculatum* at metamorphosis. Density and carbaryl reduced size of *A. maculatum* at metamorphosis, but carbaryl had a greater effect than density on body size (Table 1; Fig. 1). Statistical comparisons of growth rate produced similar results as size at metamorphosis (Tables 1 and 2). Body condition, estimated as per cent lipid, was similar for both species (Fig. 1). Per cent lipid was reduced by both density and carbaryl in *A. maculatum*, but the effect of carbaryl was generally greater than density alone (Table 1; Fig. 1).

The timing of metamorphosis was unaffected by salamander density, but was influenced by carbaryl. However, the significant postponement of metamor-

Table 1 Summary of analyses of covariance (ANCOVA) for larval period, snout-vent length (SVL), per cent lipid, growth rate, per cent survival and per cent metamorphosis for spotted salamanders (*Ambystoma maculatum*)

Response variable	Source of variation	d.f.	Mean square	*F*-value	*P*-value
Days to metamorphosis	Density	1	0.0007	0.96	0.3506
	Carbaryl	1	0.0545	70.57	<0.0001*
	Density × carbaryl	1	0.0001	0.16	0.6936
	Covariate (survival)	1	0.0003	0.37	0.5577
	Error	10	0.0008		
SVL	Density	1	0.0134	28.49	0.0003*
	Carbaryl	1	0.0572	121.07	<0.0001*
	Density × carbaryl	1	0.0017	3.54	0.0894
	Covariate (survival)	1	0.0012	2.57	0.1401
	Error	10	0.0005		
Per cent lipid	Density	1	0.0195	21.26	0.0010*
	Carbaryl	1	0.0265	28.95	0.0003*
	Density × carbaryl	1	0.0047	5.14	0.0469
	Covariate (survival)	1	0.0002	0.26	0.6224
	Error	10	0.0009		
Growth rate	Density	1	0.0231	30.20	0.0003*
	Carbaryl	1	0.2303	300.16	<0.0001*
	Density × carbaryl	1	0.0024	3.18	0.1050
	Covariate (survival)	1	0.0031	4.06	0.0717
	Error	10	0.0008		
Per cent survival	Density	1	0.1014	1.97	0.1772
	Carbaryl	2	2.1260	41.35	<0.0001*
	Density × carbaryl	2	0.1302	2.53	0.1074
	Error	18	0.0514		
Per cent metamorphosis	Density	1	0.3097	13.14	0.0021*
	Carbaryl	2	0.5621	23.85	<0.0001*
	Density × carbaryl	2	0.0724	3.07	0.0727
	Covariate (survival)	1	0.1501	6.37	0.0219
	Error	17	0.0236		

*Statistical significance after sequential Bonferroni adjustment.

phosis was species-specific. *Ambystoma maculatum* experienced a 33% increase in days to metamorphosis after exposure to carbaryl, whereas the timing of metamorphosis was similar across treatments in *A. opacum* (Tables 1 and 2; Fig. 1).

Response variables related to recruitment (per cent survival and metamorphosis) were also influenced by our experimental treatments, but the responses were strongly dependent upon species (Fig. 1). Density had no effect on the percentage of individuals of either species surviving, but carbaryl reduced survival in both species (Tables 1 and 2; Fig. 2). In *A. maculatum*, the effect of carbaryl on survival was only evident at the highest concentration (7.0 mg L^{-1}) administered. In contrast, *A. opacum* exhibited up to 97% reductions in survival at 3.5 mg L^{-1} carbaryl and 100% mortality at 7.0 mg L^{-1}. In fact, we retrieved no surviving *A. opacum* from half ($n = 4$) of the mesocosms treated with 3.5 mg L^{-1}. All *A. opacum* used in the study either died or metamorphosed, resulting in equivalent per cent survival and per cent metamorphosis in this species (Fig. 1). In contrast, 51 *A. maculatum* larvae were retrieved at the end of the experiment, all of which came from mesocosms treated with carbaryl. Of the 51 larvae retrieved, 47 were from 3.5 mg L^{-1} carbaryl treatments and four were from mesocosms treated with 7.0 mg L^{-1} carbaryl. There were no significant differences in size or lipid reserves in larvae retrieved from these treatments (in all cases, $P > 0.163$). The cumulative result of developmental delays and reduction in survival in *A. maculatum* was a reduction in per cent metamorphosis in response to carbaryl (Table 1; Fig. 2).

Effects on zooplankton

Prior to treating mesocosms with carbaryl, mean zooplankton abundance ranged from 32 to 53 individuals L^{-1}. The zooplankton communities in all mesocosms consisted primarily of Daphniidae

Table 2 Summary of analyses of covariance (ANCOVA) for larval period, snout-vent length (SVL), per cent lipid, growth rate, per cent survival and per cent metamorphosis for marbled salamanders (*Ambystoma opacum*)

Response variable	Source of variation	d.f.	Mean square	*F*-value	*P*-value
Days to metamorphosis	Density	1	0.0002	0.23	0.6485
	Carbaryl	1	0.0002	0.19	0.6722
	Density × carbaryl	1	0.0025	2.41	0.1642
	Covariate (survival)	1	<0.0001	0.04	0.8436
	Error	7	0.0010		
SVL	Density	1	0.0046	9.46	0.0179
	Carbaryl	1	0.0042	8.56	0.0221
	Density × carbaryl	1	0.0033	6.81	0.0349
	Covariate (survival)	1	<0.0001	0.02	0.8841
	Error	7	0.0005		
Per cent lipid	Density	1	0.0047	3.52	0.1026
	Carbaryl	1	0.0058	4.35	0.0754
	Density × carbaryl	1	0.0030	2.26	0.1766
	Covariate (survival)	1	0.0001	0.08	0.7799
	Error	7	0.0013		
Growth rate	Density	1	0.0070	3.47	0.1047
	Carbaryl	1	0.0061	3.02	0.1259
	Density × carbaryl	1	0.0118	5.83	0.0465
	Covariate (survival)	1	<0.0001	0.01	0.9448
	Error	7	0.0020		
Per cent survival	Density	1	0.0384	0.80	0.3843
	Carbaryl	2	4.9053	101.56	<0.0001*
	Density × carbaryl	2	0.0317	0.66	0.5312
	Error	18	0.0483		
Per cent metamorphosis	Density	1	0.0384	0.80	0.3843
	Carbaryl	2	4.9053	101.56	<0.0001*
	Density × carbaryl	2	0.0317	0.66	0.5312
	Error	18	0.0483		

*Statistical significance after sequential Bonferroni adjustment.
Note: Survival was not used as covariate in the analysis of per cent metamorphosis because all surviving *A. opacum* metamorphosed.

(Branchiopoda) and Diaptomiidae (Copepoda: Calanoida). Following carbaryl application, zooplankton abundance was significantly reduced, but this effect was dependent upon concentration (Fig. 3; time × concentration: $F = 15.78$, $P < 0.001$). Control mesocosms experienced 10–51% reductions in zooplankton, but the reduction was density-dependent suggesting that it was because of predation by salamanders (Fig. 3). However, all mesocosms treated with carbaryl experienced more pronounced reductions in zooplankton, ranging from 76 to 97%, regardless of the concentration of carbaryl added or the density of salamanders present (Fig. 3).

Discussion

Our results demonstrate that zooplankton assemblages are nearly eliminated by high, but ecologically realistic, concentrations of carbaryl and that predatory salamanders reliant upon zooplankton for food are also adversely affected. Although our study was not designed to distinguish between direct toxic effects of carbaryl on salamanders and indirect effects on salamanders via reductions in food resources, the net result of carbaryl application on the salamanders was severe. Both carbaryl and increased density negatively affected *Ambystoma* salamanders by reducing their size, growth rate, lipid reserves, survival and per cent metamorphosis. Additionally, carbaryl (but not density) increased the larval period of *A. maculatum*. The combined effects of carbaryl and increased density on per cent metamorphosis were nearly additive; when the two species were considered together, addition of 3.5 mg L^{-1} carbaryl resulted in 56% reductions in metamorphosis in the low and 92% in the high density mesocosms. However, the two stressors generally had less than additive effects on other endpoints. To place the effect of

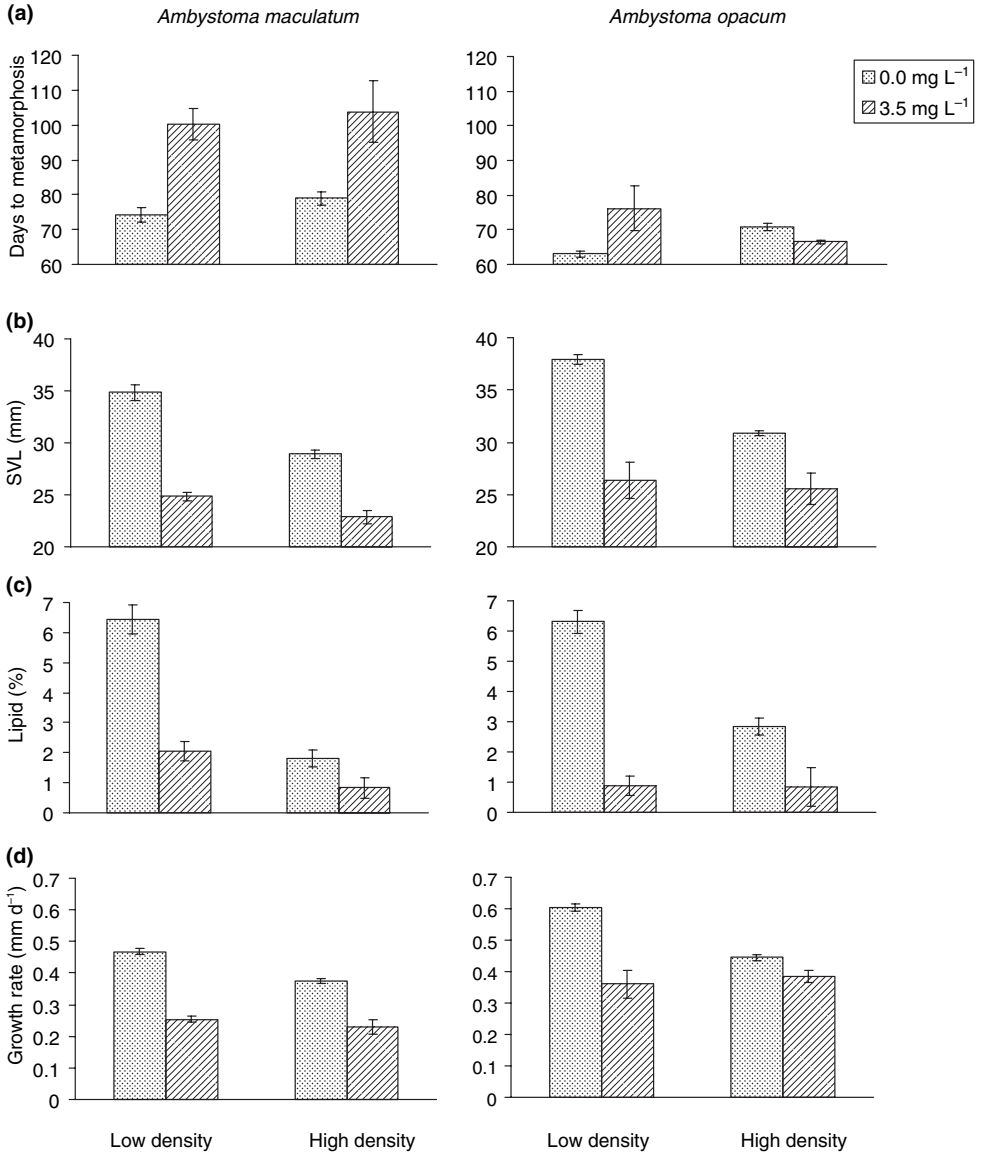

Fig. 1 Effects of carbaryl and density on larval period (a), SVL (b), lipid reserves (c) and growth rate (d) of spotted salamanders (*Ambystoma maculatum*) and marbled salamanders (*Ambystoma opacum*). Error bars represent ±1 SE.

carbaryl in perspective, the influence of 3.5 mg L^{-1} carbaryl on the salamanders was typically greater than the effect of a threefold increase in density. When carbaryl concentration was increased to 7.0 mg L^{-1}, the effect of carbaryl overshadowed all effects of density.

In some cases, it was difficult to detect differences in responses between density treatments, which may have hampered our ability to examine the additive effect of density and carbaryl on various endpoints. This appeared to be caused by carbaryl-induced reductions in survival, which narrowed the breadth of functional densities between density treatments. We initiated the experiment with densities of 12 or 36 individuals per mesocosm (low and high density, respectively), and at the end of the study this threefold breadth of densities remained in the tanks receiving no carbaryl (survival = 10.8 ± 0.48 versus 29.8 ± 0.85 in low and high density, respectively). However, in the 3.5 mg L^{-1} carbaryl treatments the range of densities decreased to less than twofold (survival = 6.0 ± 0.71 versus 11.5 ± 0.87 in low and high density, respect-

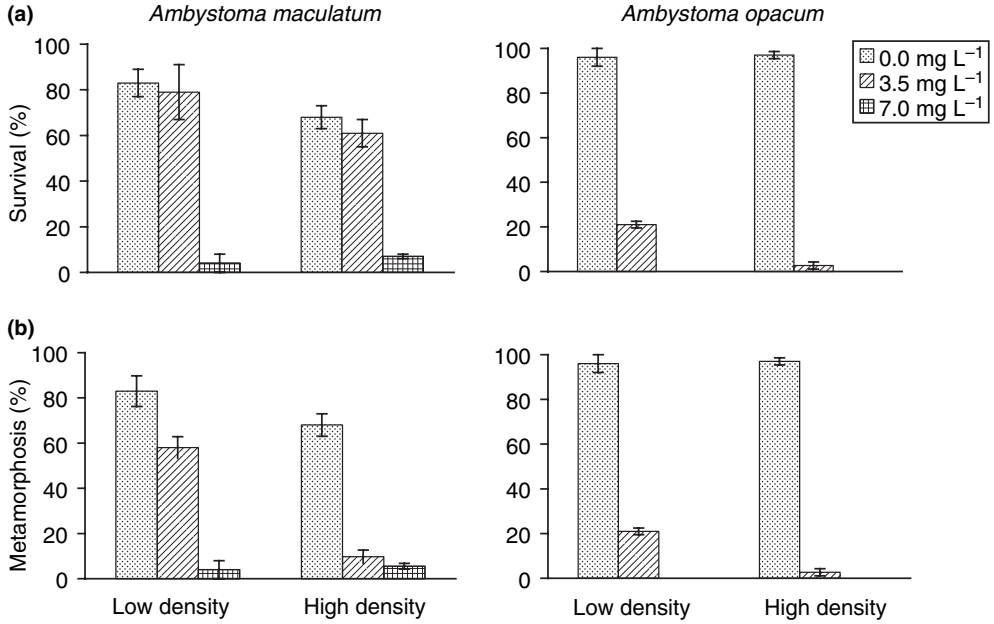

Fig. 2 Effects of carbaryl and density on survival (a) and metamorphosis (b) of spotted salamanders (*Ambystoma maculatum*) and marbled salamanders (*Ambystoma opacum*). Error bars represent ±1 SE.

ively) and in the 7.0 mg L^{-1} treatments final densities were equivalent across density treatments. Similarly, Hooper *et al.* (2003) demonstrated that the joint effects of larval density and chemical contamination on population growth rate of the midge, *Chironomus riparius* (Meigen), were less than additive. Their results suggest that short-lived pesticides may be lethal to a proportion of the population initially, resulting in fewer individuals surviving to compete for resources (Hooper *et al.*, 2003).

Our results suggest that sensitivity to chemical contamination may be highly variable among amphibian species. Although we focused our study on two closely related, sympatric ambystomatids, the responses of the two species to carbaryl varied substantially. In general, *A. opacum* was much more sensitive to carbaryl contamination than *A. maculatum*. A lower proportion of *A. opacum* survived and metamorphosed compared with *A. maculatum* in the carbaryl treatments, but the pesticide provoked a variety of

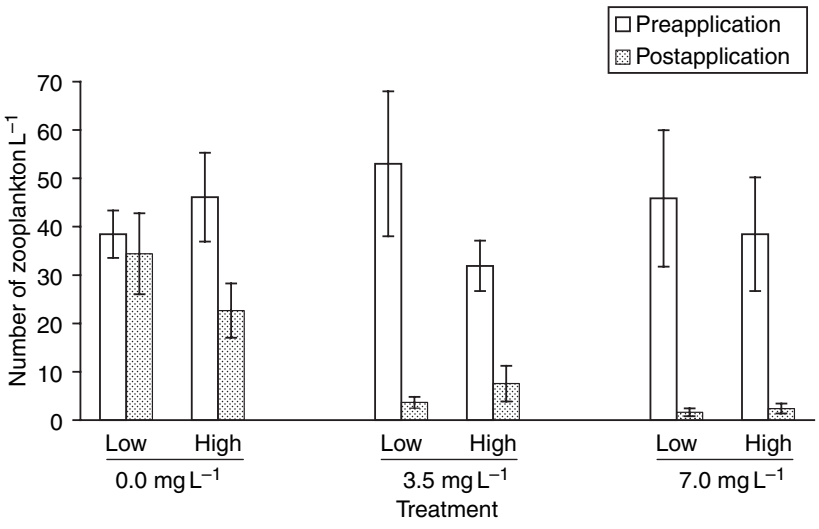

Fig. 3 Mean abundance of zooplankton per litre in tanks sampled prior to and 2 weeks after carbaryl application. Error bars represent ±1 SE.

sublethal responses in surviving *A. maculatum*. Moreover, no *A. opacum* survived the high carbaryl treatment (7.0 mg L^{-1}), but a small percentage (4%) of *A. maculatum* did. Because we used a community-approach and did not expose the two species separately, we cannot eliminate the possibility that relative ability to compete for resources differed between species, and contributed to the observed species-specific responses. Nevertheless, our results support previous studies that have demonstrated variation in tolerance to carbaryl among nine ranid species (Bridges & Semlitsch, 2000), and within a single population of *Hyla versicolor* (LeConte) (Semlitsch, Bridges & Welch, 2000). Knowledge of species-specificity in chemical tolerance is critical to identify which species are most susceptible, as well as to help explain why some species or populations suffer declines while others persist.

The observed reduction in stored lipids and growth of salamanders exposed to carbaryl and high salamander density may be of ecological importance and further illustrates the taxonomic specificity of carbaryl's effects. Because carbaryl reduces food resources of *Ambystoma* larvae, changes in per capita resources possibly resulted in the observed reductions in growth and condition. In contrast, a recent study examining the effects of carbaryl on southern leopard frog (*Rana sphenocephala* Harlan) larvae found the pesticide caused significant increases in growth and marginally significant increases in per cent lipid content (C. Bridges and W. Hopkins, unpublished data). Whereas carnivorous salamanders are negatively impacted by reductions in zooplankton, herbivorous anuran larvae sometimes prosper from carbaryl-induced reductions in invertebrate competitors for phytoplankton (Boone & Semlitsch, 2002).

Carbaryl significantly increased salamander larval period, an important life history trait that is often positively related to size at metamorphosis in salamanders. In some amphibian species, larger size at metamophosis increases survival and size at first reproduction and decreases time to first reproduction (Berven & Gill, 1983; Semlitsch, Scott & Pechmann, 1988). Further, larger size at reproductive maturity improves mating success of males and increases the size and number of eggs produced by females (Salthe, 1969; Semlitsch, 1987; Semlitsch *et al*., 1988). Thus, the timing of metamorphosis is believed to occur at an age that maximises both growth and survival (Ryan & Semlitsch, 1998). Interestingly, larval period and size at metamorphosis were not positively correlated in salamanders exposed to carbaryl. Instead, longer larval periods in *A. maculatum* resulted in smaller metamorphs, suggesting that poor resource conditions in the carbaryl-treated mesocosms increased the time it took for individuals to reach the minimum size for metamorphosis. Similar weakened relationships between size at metamorphosis and larval period have recently been noted for anuran larvae exposed to a mixture of metals and metalloids (Snodgrass *et al*., 2004), suggesting that this phenomenon is worthy of further study in amphibians exposed to contaminants.

Although sublethal effects on growth, body condition and time to metamorphosis are potentially important manifestations of carbaryl toxicity, reduction in recruitment is clearly important because it directly reduces the number of individuals that ultimately have the potential to reproduce and contribute to population-level processes. Thus, pesticide-induced declines in survival and metamorphosis could have significant impacts on local populations. In our study, carbaryl contamination increased salamander mortality four- and seven-fold in the 3.5 mg L^{-1} and 7.0 mg L^{-1} treatments, respectively. Additionally, our data suggest that increasing density may exacerbate the effect of carbaryl on survival and metamorphosis (Fig. 2). This level of mortality on aquatic life stages would likely have significant effects on terrestrial communities via reductions in salamander recruitment. Indeed, when both species are considered together metamorphosis was 1 and 23% in the 7.0 mg L^{-1} and 3.5 mg L^{-1} treatments, respectively, compared with 86% in controls.

Our study, in conjunction with others, suggests that pesticides can alter amphibian communities and these effects can be further influenced by natural factors such as density (this study), competition and predation (Boone & Semlitsch, 2001; Relyea & Mills, 2001). This study, which is one of the few to examine the influence of such factors on salamanders, suggests that density can influence the effects that chemical contaminants have on recruitment of ambystomatids to the terrestrial environment. Because carbaryl affects organisms similarly to other cholinesterase-inhibiting pesticides (Rand, 1995), and because the density of salamander assemblages can vary widely in nature, other pesticides may have similar impacts on salamander communities. Based on accumulating evi-

dence that pesticide effects are often indirect (Fleeger, Carman & Nisbet, 2003), influenced by other biotic and abiotic variables and highly variable among species, single-factor laboratory studies may not be predictive of the real-world consequences of pesticide use. Thus, the complexity of natural systems must be considered in future studies attempting to determine the effects of pesticides on amphibian communities and how these effects might relate to amphibian decline.

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References

Barinaga M. (1990) Where have all the froggies gone? *Science*, **247**, 1033–1034.

Berven K.A. & Gill D.E. (1983) Interpreting geographic variation in life-history traits. *American Zoologist*, **23**, 85–97.

Beyers D.W. & Meyers O.B. (1996) Use of meta-analysis to predict degradation of carbaryl and malathion in freshwater for exposure assessment. *Human and Ecological Risk Assessment*, **2**, 3366–3380.

Blaustein A.R. & Wake D.B. (1990) Declining amphibian populations: a global phenomenon? *Trends in Ecology and Evolution*, **5**, 203–204.

Boone M.D. & Bridges C.M. (1999) The effect of temperature on the potency of carbaryl for survival of tadpoles of the green frog (*Rana clamitans*). *Environmental Toxicology and Chemistry*, **18**, 1482–1484.

Boone M.D. & James S.M. (2003) Interactions of an insecticide, herbicide, and natural stressors in amphibian community mesocosms. *Ecological Applications*, **13**, 829–841.

Boone M.D. & Semlitsch R.D. (2001) Interactions of an insecticide with larval density and predation in experimental amphibian communities. *Conservation Biology*, **15**, 228–238.

Boone M.D. & Semlitsch R.D. (2002) Interactions of an insecticide with competition and pond drying in amphibian communities. *Ecological Applications*, **12**, 307–316.

Bridges C.M. (1997) Tadpole swimming performace and activity affected by acute exposure to sublethal levels of carbaryl. *Environmental Toxicology and Chemistry*, **16**, 1935–1939.

Bridges C.M. (1999) *The Effects of a Chemical Stressor on Amphibian Larvae: Individual, Population and Species Level Responses*. University of Missouri, Columbia, Missouri.

Bridges C.M. (2000) Long-term effects of pesticide exposure at various life stages of the southern leopard frog (*Rana sphenocephala*). *Archives of Environmental Contamination and Toxicology*, **39**, 91–96.

Bridges C.M. & Boone M.D. (2003) The interactive effects of UV-B and insecticide exposure on tadpole survival, growth and development. *Biological Conservation*, **113**, 49–54.

Bridges C.M. & Semlitsch R.D. (2000) Variation in pesticide tolerance of tadpoles among and within species of Ranidae and patterns of amphibian decline. *Conservation Biology*, **14**, 1490–1499.

Capel P.D., Larson S.J. & Winterstein T.A. (2001) The behaviour of 39 pesticides in surface waters as a function of scale. *Hydrological Processes*, **15**, 1251–1269.

Carey C., Cohen N. & Rollins-Smith L. (1999) Amphibian declines: an immunological perspective. *Developmental and Comparative Immunology*, **23**, 459–472.

Corn P.S. (1994) What we know and do not know about amphibian declines in the West. In: *Sustainable Ecological Systems: Implementing an Ecological Approach to Land Management*. General Technical Report RM-247 (Eds W.W. Covington & L.F. DeBano), pp. 59–67. U.S. Forest Service, Fort Collins, Colorado.

Cox C. (1993) Carbaryl. *Journal of Pesticide Reform*, **13**, 31–36.

Davidson C., Shaffer H.B. & Jennings M.R. (2001) Declines of the California red-legged frog: climate, UV-B, habitat, and pesticide hypotheses. *Ecological Applications*, **11**, 464–479.

Dodd C.K. (1997) Imperiled amphibians: a historical perspective. In: *Aquatic Fauna in Peril: the Southeastern Perspective* (Eds G.W. Benz & D.E. Collins), pp. 165–200. Southeastern Aquatic Research Institute, Decatur, Georgia.

Fleeger J.W., Carman K.R. & Nisbet R.M. (2003) Indirect effects of contaminants in aquatic ecosystems. *The Science of the Total Environment*, **317**, 207–233.

Gilliom R.J. (2001) Pesticides in hydrologic system – what do we know and what's next? *Hydrological Processes*, **15**, 3197–3201.

Hanazato T. & Yasuno M. (1987) Effects of a carbamate insecticide carbaryl on the summer phytoplankton and zooplankton communities in ponds. *Environmental Pollution*, **48**, 145–159.

Hanazato T. & Yasuno M. (1990) Influence of *Chaoborus* density on the effects of an insecticide on zooplankton communities in ponds. *Hydrobiologia*, **194**, 183–197.

Hooper H.L., Sibly R.M., Maund S.J. & Hutchinson T.H. (2003) The joint effects of larval density and 14C-cypermethrin on life history and population growth rate of the nidge *Chironomus riparius*. *Journal of Applied Ecology*, **40**, 1049–1089.

Hopkins W.A., Snodgrass J., Roe J.H., Jackson B.P., Gariboldi J.C. & Congdon J.D. (2000) Detrimental effects associated with trace element uptake in lake chubsuckers, *Erimyzon sucetta* exposed to polluted sediments. *Archives of Environmental Contamination and Toxicology*, **39**, 193–199.

Hopkins W.A., Snodgrass J.W., Roe J.H., Staub B.P., Jackson B.P. & Congdon J.D. (2002) Effects of food ration on survival and sublethal responses of lake chubsuckers (*Erimyzon sucetta*) exposed to coal combustion wastes. *Aquatic Toxicology*, **57**, 906–913.

Hopkins W.A., Staub B.P., Snodgrass J.W., DeBiase A., Taylor B., Roe J.H., Jackson B.P. & Congdon J.D. (2004) Response of benthic fish exposed to contaminants in outdoor mesocosms: examining the ecological relevance of previous laboratory toxicity tests. *Aquatic Toxicology*, **68**, 1–12.

Houlahan J.E., Findlay C.S., Schmidt B.R., Meyer A.H. & Kuzmin S.L. (2000) Quantitative evidence for global amphibian population declines. *Nature*, **404**, 752–755.

Marian M.P., Arul V. & Pandian T.J. (1983) Acute and chronic effects of carbaryl on survival, growth and metamorphosis in the bullfrog (*Rana tigrina*). *Archives of Environmental Contamination and Toxicology*, **12**, 271–275.

Mayer F.L. & Ellersieck M.R. (1986) *Manual of Acute Toxicity: Interpretation and Data Base for 410 Chemicals and 66 Species of Freshwater Animals*. Fisheries and Wildlife Service, Washington, D.C.

Mills N.E. & Semlitsch R.D. (2004) Competition and predation mediate the indirect effects of an insecticide on southern leopard frogs. *Ecological Applications*, **14**, 1041–1054.

Norris L.A., Lorz H.W. & Gregory S.V. (1983) *Influence of Forest and Range Land Management on Anadromous Fish Habitat in Western North America: Forest Chemicals*. United States Forest Service, Portland, Oregon.

Parris M.J. & Semlitsch R.D. (1998) Assymmetric competition in larval amphibian communities: conservation implications for the northern crawfish frog, *Rana areolata circulosa*. *Oecologia*, **116**, 219–226.

Peterson H.G., Boutin C., Martin P.A., Freemark K.E., Ruecker N.J. & Moody M.J. (1994) Aquatic phytotoxicity of 23 pesticides applied at expected environmental concentrations. *Aquatic Toxicology*, **28**, 275–292.

Rand G.M. (1995) *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*. Taylor & Francis, Bristol, Pennsylvania.

Relyea R.A. (2003) Predator cues and pesticides: a double dose of danger for amphibians. *Ecological Applications*, **13**, 1515–1521.

Relyea R.A. & Mills N. (2001) Predator-induced stress makes the pesticide carbaryl more deadly to gray treefrog tadpoles (*Hyla versicolor*). *Proceedings National Academy of Science*, **98**, 2491–2496.

Ryan R.J. & Semlitsch R.D. (1998) Intraspecific heterochrony and life history evolution: decoupling somatic and sexual development in a facultatively paedomorphic salamander. *Proceedings of the National Academy of Science*, **95**, 5643–5648.

Salthe S.N. (1969) Reproductive modes and the number and sizes of ova in the urodeles. *American Midlands Naturalist*, **81**, 467–490.

SAS Institute (1999–2004) *SAS version 9.0*. SAS Institute, Cary, North Carolina.

Scott D.E. & Fore M.R. (1995) The effect of food limitation on lipid levels, growth, and reproduction in the marbled salamander, *Ambystoma opacum*. *Herpetologica*, **51**, 462–471.

Semlitsch R.D. (1987) Density-dependent growth and fecundity in the paedomorphic salamander *Ambystoma talpoideum*. *Ecology*, **68**, 1003–1008.

Semlitsch R.D. & Walls S.C. (1993) Competition in two species of larval salamanders: a test of geographic variation in competitive ability. *Copeia*, **1993**, 587–595.

Semlitsch R.D., Scott D.E. & Pechmann J.H.K. (1988) Time and size at metamorphosis related to adult fitness in *Ambystoma opacum*. *Ecology*, **69**, 184–192.

Semlitsch R.D., Bridges C.M. & Welch A.M. (2000) Genetic variation and fitness tradeoff in the tolerance of gray treefrog (*Hyla versicolor*) tadpoles to the insecticide carbaryl. *Oecologia*, **125**, 179–185.

Snodgrass J.W., Hopkins W.A., Broughton J., Gwinn D., Baionno J.A. & Burger J. (2004) Species specific responses of developing anurans to coal combustion wastes. *Aquatic Toxicology*, **66**, 171–182.

Travis J. (1983) Variation in growth and survival of *Hyla gratiosa* larvae in experimental enclosures. *Copeia*, **1983**, 232–237.

United States Environmental Protection Agency (1992) Pesticide industry sales and usage: 1990 and 1991 market estimates. In: *Pesticides and Toxic Substances*, pp. 1–32. EPA, Washington, D.C.

Wake D.B. (1991) Declining amphibian populations. *Science*, **253**, 860.

Wilbur H.M. (1980) Complex life cycles. *Annual Review of Ecological Systems*, **11**, 67–93.

Wilbur H.M. (1997) Experimental ecology of food webs: complex systems in temporary ponds. *Ecology*, **68**, 1437–1452.

Wilbur H.M. & Collins J.P. (1973) Ecological aspects of amphibian metamorphosis. *Science*, **182**, 1305–1314.

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