

INTERACTIONS OF AN INSECTICIDE, HERBICIDE, AND NATURAL STRESSORS IN AMPHIBIAN COMMUNITY MESOCOSMS

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Abstract. Amphibians developing in wetlands embedded within or near agricultural lands may frequently encounter chemical mixtures. The objectives of our study were to determine the effects that post-application concentrations of an insecticide (carbaryl) and an herbicide (atrazine) have on body mass, development, and survival of two anuran species (southern leopard frog, *Rana sphenocephala*; American toad, *Bufo americanus*) and two caudate species (spotted salamander, *Ambystoma maculatum*; small-mouthed salamander, *A. texanum*) reared in outdoor cattle tank mesocosms. In one experiment, we manipulated tadpole density (low or high), carbaryl exposure (0, 3.5, 7.0 mg/L), and atrazine exposure (0 or 200 µg/L) to test for effects on development, mass, and survival of larvae. In a second experiment, we manipulated pond hydroperiod (constant or drying), carbaryl exposure (0 or 5 mg/L), and atrazine exposure (0 or 200 µg/L) to test for effects on mass, time, and survival to metamorphosis. Salamanders were virtually eliminated in carbaryl treatments, indicating that at realistic levels, this insecticide could cause population declines for salamanders in contaminated habitats. Carbaryl also had negative effects on toad survival. Exposure to atrazine had negative effects on body size, development, and time to metamorphosis in anuran species, which were associated with reduced chlorophyll levels. Both chemicals interacted significantly with density or hydroperiod, indicating that the environmental conditions could influence the impact of a contaminant. A significant atrazine-by-carbaryl interaction resulted in smaller and less developed spotted salamander larvae than in control ponds. Atrazine exposure, however, appeared to moderate negative effects of carbaryl for spotted salamanders. Our research suggests that important changes in the community's food web result from chemical exposure, which influence the susceptibility of amphibian species to contaminants.

Key words: *Ambystoma*; amphibian decline; anurans; atrazine; *Bufo*; carbaryl; chemical mixtures; herbicide; insecticide; *Rana*; salamanders.

INTRODUCTION

Environmental conditions can determine species distribution, abundance, and diversity and can influence the outcome of competitive and predatory interactions. In early ecological experiments, Park (1948) demonstrated in the laboratory and Connell (1961) in the field that the change in biotic (e.g., presence of a parasite) and abiotic (e.g., inundation) factors could reverse the outcome of competition. In amphibian communities, the role of competition, predation, and abiotic factors has been examined experimentally and these studies demonstrate how these factors, alone and in combination, influence the structure of amphibian communities (e.g., Wilbur 1972, 1987, Morin 1981, 1983).

In human-dominated landscapes, many novel stressors (e.g., exotic species, thermal pollution, chemical contaminants) can alter community structure. Because applied research on chemical contaminants has focused on single factors, the effects that multiple anthropo-

genic stressors have on communities is largely unexamined. If we fail to test multifactor hypotheses, we risk proposing solutions that are too simplistic, thus failing to solve environmental problems (Hilborn and Stearns 1982) at the cost of population and species extinction. Single-factor explanations simply may not be sufficient to explain widespread phenomenon such as amphibian declines.

Amphibians breeding in wetlands embedded within or near agricultural lands may experience natural stresses from competition and predation in ephemeral environments, in addition to single or multiple chemical stressors. There has been limited research on the effects of chemical mixtures on plankton and fish (Fairchild et al. 1994) and on amphibian communities (Britson and Threlkeld 1998, 2000). The weakness of some mixture studies is that the interactive effects of single chemicals in all possible combinations are not evaluated, therefore the applicability of such studies is unclear. Evaluation of contaminants occurring in mixtures in the field has proven useful for common mixtures (e.g., coal ash; Hopkins et al. 1998, 2000, Rowe et al. 2001), even if components of effects and underlying mechanisms are initially uncertain. However, such

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studies do suggest that chemical interactions could be important at expected environmental levels.

The most extensive examination of the effects of a single pesticide on amphibians has been with the insecticide carbaryl, a carbamate neurotoxin that appears to have low toxicity on amphibians (Bridges 1997, Boone and Semlitsch 2001, 2002). However, this short-lived chemical can alter survival, body mass, and time to metamorphosis for some species (Bridges 2000, Boone and Semlitsch 2001, 2002, Boone et al. 2001, Mills 2002) at realistic, post-application levels (≤ 4.8 mg/L; Norris et al. 1983, Peterson et al. 1994). Field studies suggest that the main effect of this insecticide for amphibian communities is indirect rather than direct (Mills 2002). The indirect effect occurs mainly by reducing or eliminating zooplankton populations, which are sensitive to insecticides (Mayer and Ellersieck 1986, Hanazato and Yasuno 1990). A reduction in zooplankton causes an algal bloom that can increase survival and mass at metamorphosis for herbivores (e.g., anurans; Boone and Semlitsch 2002, Mills 2002). Conversely, carnivorous salamander larvae that feed on zooplankton and invertebrates may be negatively influenced by insecticide exposure through a reduction in food resources, although toxicology studies with caudates are rare.

While studies with carbaryl are important because they establish causal links between the effects of a pesticide on larval amphibian communities, they may underestimate the impact, because chemicals are often applied in mixtures of insecticides and herbicides in agricultural systems. The herbicide atrazine is widely used to control row-crop weeds, primarily in corn (Solomon et al. 1996). Atrazine inhibits photosynthesis and can reduce primary production, although this effect is reversible (Solomon et al. 1996). While it is generally accepted that surface waters seldom exceed 50 $\mu\text{g/L}$ (Solomon et al. 1996), small wetlands within agricultural areas may frequently exceed this amount to levels up to and greater than 500 $\mu\text{g/L}$ (de Noyelles et al. 1982, Howe et al. 1998). Acute toxicity from atrazine is unlikely for larval amphibians, because LC50s (the lethal concentration causing 50% mortality in a population) exceed expected environmental concentrations by orders of magnitude (e.g., LC50 of northern leopard frog [*Rana pipiens*], 47.6 mg/L; Howe et al. 1998). At concentrations above levels expected in the field, however, deformities and reduced feeding have been documented (Allran and Karasov 2001). For the above reasons, realistic sublethal concentrations or indirect effects may be more relevant to amphibian communities.

Phytoplankton, the food resources for anurans and zooplankton, are diminished by atrazine by 50–200 $\mu\text{g/L}$ (Fairchild et al. 1998, Diana et al. 2000), although recovery of phytoplankton is common (Diana et al. 2000). At these levels, phytoplankton should be reduced for a time and herbivorous amphibians may be

affected by lower food resources. Diana et al. (2000) found that at 200 $\mu\text{g/L}$ atrazine, gray treefrogs (*Hyla versicolor*) had a smaller size and mass at metamorphosis. Atrazine, therefore, can affect anurans at these concentrations, and we anticipated that the presence of an herbicide would reduce food resources for anurans even if an insecticide could indirectly increase them. The objectives of our study were to determine the effects that expected environmental concentrations of an insecticide (carbaryl) and an herbicide (atrazine) have on body mass, development, and survival of two anuran (southern leopard frog, *Rana sphenoccephala*; American toad, *Bufo americanus*) and two caudate (spotted salamander, *Ambystoma maculatum*; small-mouthed salamander, *A. texanum*) species in outdoor cattle tank mesocosms.

METHODS

We collected three egg masses of southern leopard frogs (*Rana sphenoccephala*) and 21 egg masses of spotted salamanders (*Ambystoma maculatum*) on 14 March and 27 March 2000, respectively, from the Basket Wildlife Area (Boone County, Ashland, Missouri, USA). We collected seven egg masses of the American toad (*Bufo americanus*) from the Forum Nature Area (Boone County, Columbia, Missouri, USA) and ~30 egg masses of small-mouth salamanders (*Ambystoma texanum*; females lay multiple groups of eggs) from the Baskett Wildlife Area on 30 March and 9 April 2001, respectively. Eggs were hatched in the laboratory at 23–25°C and held until all larvae were free-swimming. We mixed clutches within each species before use in our experiments to homogenize genetic variation.

We created aquatic communities in polyethylene cattle tank ponds (1.85 m in diameter; 1480 L volume) by adding 1000 L of tap water, 1 kg of leaf litter from a mixed, deciduous forest, and plankton from natural ponds (500 mL of plankton per pond at six different times) in mid-March 2000 and 2001. Plankton populations were well established in the ponds by the time larvae were introduced. The ponds were located in a fenced field at the University of Missouri Research Park in Columbia (Boone County), Missouri. Screen-mesh lids covered each pond to exclude incidental predators and anuran colonists. Use of outdoor, pond mesocosms 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 daily variation in temperature that would be present in a typical pond (Rowe and Dunson 1994). In all analyses, ponds represented the experimental unit to avoid problems of pseudoreplication (Hurlbert 1984).

Experiment I: effects of competition, atrazine, and carbaryl on larval amphibians

We experimentally manipulated three factors in a fully crossed design with three replicates (36 ponds):

competition via initial anuran density (low [20 tadpoles/1000 L] or high [60 tadpoles/1000 L]); carbaryl concentration (0, 3.5, or 7.0 mg/L); and atrazine concentration (0 or 200 $\mu\text{g/L}$). In addition, competition controls (each species alone with two densities for anurans and one density for caudates, replicated three times; nine ponds) were also established to determine if rearing species together had an effect on body mass, development, or survival. Twelve spotted salamander (*Ambystoma maculatum*) larvae were added to each pond on 28 March 2000; therefore, density effects were not determined for this species. Southern leopard frog (*Rana sphenocephala*) tadpoles were added to ponds on 4 April 2000 (day 0). We added carbaryl as liquid Sevin (21.3% carbaryl; Ortho, Columbus, Ohio, USA) at a nominal concentration of 3.5 mg/L (16.4 g Sevin) or 7.0 mg/L (32.9 g Sevin) and atrazine as liquid Aatrex (40.8% atrazine, Novartis, Greensboro, North Carolina, USA) at a nominal concentration of 200 $\mu\text{g/L}$ (490 mg Aatrex) on 21 April 2000 (day 17). We selected the chemical level based on post-application, expected environmental concentrations (≤ 4.8 mg/L carbaryl and ≤ 270 $\mu\text{g/L}$ atrazine; Norris et al. 1983, Peterson et al. 1994). The chemical treatment was applied by mixing the chemical(s) with 5 L of pond water and then pouring the mixture evenly across the pond surface with a watering can between 1000 and 1100 hours Central Standard Time (pond water: pH 7.9 ± 0.01 , $14.6 \pm 0.06^\circ\text{C}$). We added 5 L of uncontaminated pond water to control ponds to mimic the disturbance of chemical application. We did not stir pond water to minimize the potential of an algal bloom and because direct overspraying in the environment would not involve vigorous mixing. We took three 2-L samples from each of three ponds that were exposed to 7.0 mg/L carbaryl (and no atrazine) at 1, 24, 48, and 96 h following carbaryl exposure; at each time we combined samples from ponds and refrigerated 20 mL in a glass vial for chemical analyses. We also took three 2-L samples from each of three ponds exposed to 200 $\mu\text{g/L}$ atrazine (and no carbaryl) at 1, 15, and 57 d following atrazine exposure; on each date, we combined these samples and refrigerated 1 L in a plastic bottle for chemical analyses. The half-life was ~ 4.5 d for carbaryl (based on linear regression; 1 h, 7.0 mg/L; 24 h, 5.5 mg/L; 48 h, 4.5 mg/L; 96 h, 4.2 mg/L) and 34 d for atrazine (based on linear regression; 1 d, 207 $\mu\text{g/L}$; 15 d, 200 $\mu\text{g/L}$; 57 d, 6.4 $\mu\text{g/L}$; Mississippi State Chemical Laboratory; high performance liquid chromatography [HPLC]).

The experiment was terminated over 30 May–1 June 2000 (days 56–58) before most individuals had reached metamorphosis. At the end of our experiment, we drained ponds and removed all remaining tadpoles and salamander larvae. We determined mean body mass, developmental stage (for anurans, Gosner 1960; for salamanders, Donavan 1980), snout–vent length (SVL; salamander larvae only), and pond survival for each

species in each pond (i.e., each pond represented an experimental unit). Analyses for effects of carbaryl, atrazine, density (for tadpoles), and all interactions on mass, developmental stage, and SVL (salamander larvae only; mass, stage, and SVL comprised the “multivariate response”) were performed using a multivariate analysis of covariance (MANCOVA; SAS 1988) for ponds containing both species. We used survival as a covariate for analyses of mass, developmental stage, and SVL (salamander larvae only), because there were significant differences in survival among treatment groups. Univariate analyses of covariance (ANCOVA) were used to determine which responses were the strongest contributors to the multivariate effects. Survival was analyzed with an analysis of variance (ANOVA). Because few salamanders survived in ponds exposed to 7.0 mg/L carbaryl (see *Results*), analysis of mass, stage, and SVL only included data from ponds exposed to 0 and 3.5 mg/L carbaryl for salamanders, due to problems of missing cells. We also used ANOVA to determine if there were differences in mass, SVL (salamander larvae only), developmental stage, or survival between control ponds containing both species and control ponds containing only one species. To normalize data and stabilize variances, we angularly transformed all proportion data, log transformed mass and SVL, and used a ranking procedure (PROC RANK; SAS 1988) on developmental stage data before analyses (Snedecor and Cochran 1980). Conservative pairwise comparisons for significant main effects were performed using Scheffé’s multiple comparison tests (Snedecor and Cochran 1980).

Experiment II: effects of hydroperiod, atrazine, and carbaryl on amphibians reared through metamorphosis

We experimentally manipulated three factors in a fully crossed design with four replicates (32 ponds): hydroperiod (constant or drying with a movable standpipe), exposure to carbaryl (0 or 5.0 mg/L), and exposure to atrazine (0 or 200 $\mu\text{g/L}$). Because Experiment I indicated that rearing anurans and salamanders together did not influence our measured endpoints (see *Results*), we did not include competition controls in this experiment. Twelve small-mouthed salamander (*Ambystoma texanum*) larvae and 45 American toad (*Bufo americanus*) tadpoles were added to each pond on 16 April 2001 (day 0). We added carbaryl as liquid Sevin (22.5% carbaryl) at a nominal concentration of 5.0 mg/L (22.2 g Sevin; GardenTech, Lexington, Kentucky, USA) and atrazine as liquid (i.e., Aatrex; 40.8% atrazine) at a nominal concentration of 200 $\mu\text{g/L}$ (490 mg Aatrex) on 24 April 2001 (day 8; pond water: pH 7.7 ± 0.03 , $13.3 \pm 0.04^\circ\text{C}$). We took three 2-L samples from each of three ponds that were exposed to 5.0 mg/L carbaryl (and no atrazine) at 1 and 48 h following carbaryl exposure; at each time we combined samples from ponds and refrigerated 20 mL in a glass vial for

chemical analyses. We also took three 2-L samples from each of three ponds exposed to 200 $\mu\text{g/L}$ atrazine (and no carbaryl) 1 d following atrazine exposure to confirm the atrazine level; we combined water from the ponds and refrigerated 1 L in a plastic bottle for chemical analyses. Water analyses indicated that carbaryl had a half-life of ~ 3 d (based on linear regression; 1 h, 5.3 mg/L; 48 h, 3.4 mg/L) and confirmed the atrazine concentration (1 d, 197 $\mu\text{g/L}$; Mississippi State Chemical Laboratory, HPLC analysis).

We made daily searches for metamorphosed toads or salamanders. On 13 July 2001 (day 88), we drained ponds and removed all remaining metamorphs and larvae. Toad and salamander metamorphs were measured for mass and time to metamorphosis, and survival was determined for each species in each pond; pond means served as the experimental unit. Analyses on toads for effects of carbaryl, atrazine, hydroperiod, and all interactions on mass and time to metamorphosis (mass and time represent the "multivariate response") were performed using a MANCOVA (SAS 1988) with survival as the covariate. Univariate ANCOVAs were used to determine which responses were the strongest contributors to the multivariate effects. Survival was analyzed separately with ANOVA. For salamanders, the effects of carbaryl, atrazine, hydroperiod, carbaryl by hydroperiod, and atrazine by hydroperiod interactions on mass and time to metamorphosis were analyzed with a MANCOVA; survival was used as the covariate. All interactions could not be analyzed for salamanders due to missing cells resulting from no survival in ponds exposed to both carbaryl and atrazine treatments. Univariate ANCOVAs were used to determine which responses were the strongest contributors to the multivariate effects. To normalize data and stabilize variances, we angularly transformed all proportion data and log transformed mass and time to metamorphosis before analyses (Snedecor and Cochran 1980).

Chlorophyll content

In both studies, we estimated the standing crop of chlorophyll in the water column by taking a 1-L composite sample (sampled from three 2-L samples) from each pond. 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 and then analyzed by fluorometry (Greenberg et al. 1992). Water samples were taken on four dates in 2000 (before chemical application on 19 April [day 15] and after chemical application on 26 April [day 22], 3 May [day 29], and 17 May [day 42]) and on two dates in 2001 (before chemical application on 24 April 2001 [day 8] and after chemical application on 13 June [day 50]). Chlorophyll data were analyzed with a repeated-measures ANOVA to determine the main effects and interactions of time, carbaryl, atrazine, and density (Experiment I) or hydroperiod (Experiment II).

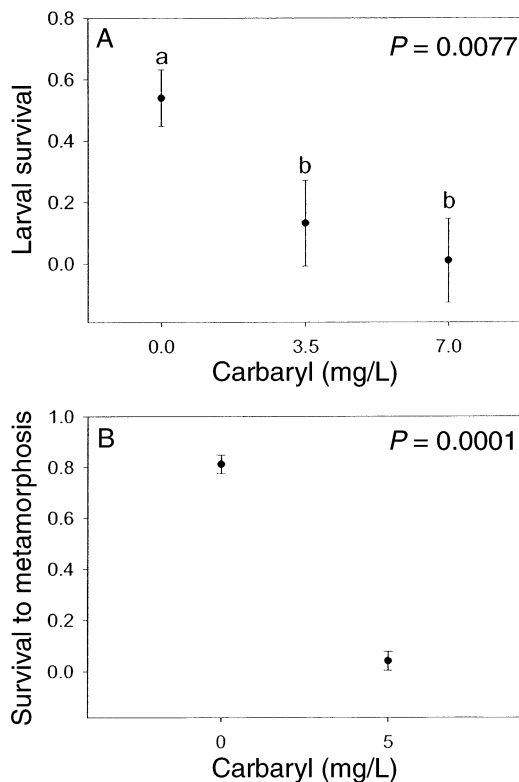


FIG. 1. Proportion of (A) spotted salamander (*Ambystoma maculatum*) larvae in Experiment I and (B) small-mouthed salamander (*A. texanum*) metamorphs in Experiment II that survived across carbaryl treatments. All amphibians used in this study were collected in Boone County, Missouri, USA. Error bars represent ± 1 SE. Different lowercase letters above the data points indicate significant differences among treatments according to Scheffé's multiple comparison tests.

RESULTS

Experiment I: effects of competition, atrazine, and carbaryl on larval amphibians

Spotted salamander survival was significantly reduced by carbaryl exposure (Fig. 1A, Table 1). Multivariate analyses on salamanders indicated that carbaryl exposure and the interaction of carbaryl by atrazine exposure negatively affected mass, SVL, and developmental stage (Table 2). The carbaryl multivariate effect on larvae that survived can be attributed to its influence on all three responses resulting in significantly reduced mass (Fig. 2A), reduced SVL (Fig. 2B), and delayed development (Fig. 2C) for larvae in ponds exposed to 3.5 mg/L carbaryl compared to controls. Atrazine exposure did not influence the responses we measured for the spotted salamanders, however the significant interaction of carbaryl and atrazine indicates that the presence of atrazine ameliorated the carbaryl treatment to some degree (Tables 1 and 2; Fig. 3). This carbaryl by atrazine interaction can be ascribed to its effect on SVL (Fig. 3B); however, body mass and developmental stage of salamanders showed a similar

TABLE 1. Summary of univariate analyses of covariance (ANCOVA) of body mass, snout-vent length (SVL; for salamanders only), developmental stage (Gosner for anurans, Donovan for caudates), and larval survival for spotted salamanders (*Ambystoma maculatum*) and southern leopard frogs (*Rana sphenoccephala*) from Experiment I.

Response variable	Source of variation	Sum of squares	df	F	P
Spotted salamander					
Mass	Carbaryl	1.8713	1	9.91	0.0084
	Carbaryl × atrazine	0.8290	1	4.39	0.0580
	Error	2.2648	12		
SVL	Carbaryl	0.2885	1	13.70	0.0030
	Carbaryl × atrazine	0.1446	1	6.87	0.0224
	Error	0.2526	12		
Developmental stage	Carbaryl	131.1140	1	5.91	0.0316
	Carbaryl × atrazine	102.1678	1	4.61	0.0530
	Error	266.0944	12		
Survival	Carbaryl	2.9923	2	5.86	0.0077
	Atrazine	0.0162	1	0.06	0.8029
	Carbaryl × atrazine	0.0053	2	0.01	0.9897
	Error	6.8981	27		
Southern leopard frog					
Mass	Carbaryl	0.5088	2	19.79	0.0001
	Atrazine	0.1303	1	10.13	0.0052
	Density	2.1677	1	168.51	0.0001
	Density × carbaryl	0.0139	2	0.54	0.5928
	Error	0.2315	18		
Developmental stage	Carbaryl	32.1010	2	0.45	0.6462
	Atrazine	60.9289	1	1.70	0.2089
	Density	2018.9690	1	56.29	0.0001
	Density × carbaryl	317.7967	2	4.43	0.0273
	Error	645.6355	18		
Survival	Carbaryl	0.1477	2	0.98	0.3947
	Atrazine	0.2249	1	2.97	0.1009
	Density	0.2492	1	3.29	0.0853
	Carbaryl × atrazine	0.1120	2	0.74	0.4901
	Density × carbaryl	0.1686	2	1.11	0.3485
	Density × atrazine	0.4591	1	6.07	0.0235
	Density × carbaryl × atrazine	0.0152	2	0.10	0.9050
	Error	1.4372	19		

Note: Statistics for sources of variation that were significant according to the MANCOVA for mass, SVL, and developmental stage are reported.

TABLE 2. Multivariate analysis of covariance (MANCOVA) for treatment effects and interactions on larval mass, developmental stage, and snout-vent length (SVL; for salamanders only) for larvae of spotted salamanders (*Ambystoma maculatum*) and southern leopard frogs (*Rana sphenoccephala*) from Experiment I.

Source of variation	Wilks' lambda	df	F	P
Spotted salamander				
Covariate (survival)	0.9016	3, 10	0.36	0.7808
Carbaryl	0.3727	3, 10	5.61	0.0162
Atrazine	0.8159	3, 10	0.75	0.5457
Carbaryl × atrazine	0.4663	3, 10	3.82	0.0466
Southern leopard frog				
Covariate (survival)	0.7462	2, 17	2.89	0.0831
Carbaryl	0.2847	4, 34	7.43	0.0002
Atrazine	0.6367	2, 17	4.85	0.0216
Density	0.0895	2, 17	86.43	0.0001
Carbaryl × atrazine	0.8135	4, 34	0.92	0.4614
Density × carbaryl	0.5822	4, 34	2.64	0.0506
Density × atrazine	0.8864	2, 17	1.09	0.3588
Density × carbaryl × atrazine	0.8292	4, 34	0.83	0.5127

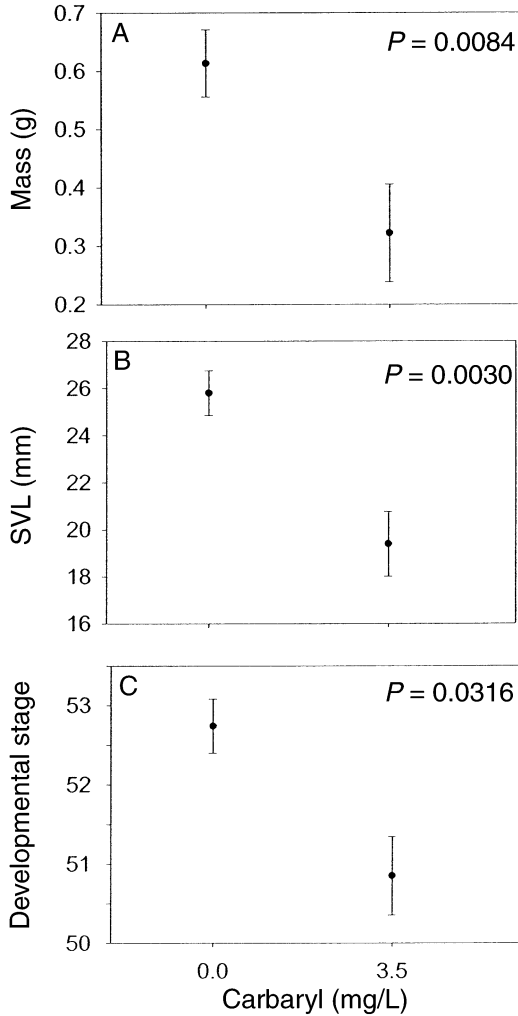


FIG. 2. (A) Mass, (B) snout-vent length (SVL), and (C) developmental stage of spotted salamander (*Ambystoma maculatum*) larvae at the end of Experiment I across carbaryl treatments. Error bars represent ± 1 SE.

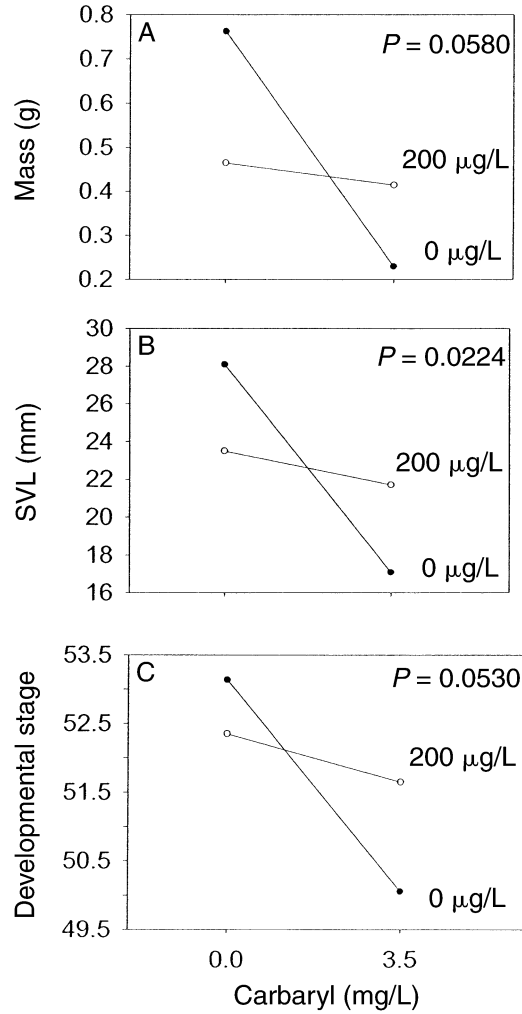


FIG. 3. (A) Mass, (B) snout-vent length (SVL), and (C) developmental stage of spotted salamander (*Ambystoma maculatum*) larvae at the end of Experiment I for salamanders exposed or not exposed to atrazine across carbaryl treatments.

trend and likely contribute to the multivariate response (Fig. 3A and C).

Survival of leopard frogs was not significantly influenced by either chemical alone (Table 1). However, leopard frog survival was significantly influenced by an atrazine by density interaction with the greatest survival in high-density control ponds and the lowest in high-density ponds exposed to atrazine; tadpoles in low-density ponds were the least affected by atrazine exposure at either concentration (Fig. 4). The multivariate responses of leopard frogs were significantly affected by carbaryl exposure, atrazine exposure, and initial tadpole density (Table 2). Leopard frog mass significantly increased with carbaryl exposure and decreased with atrazine exposure compared to controls (Table 1; Fig. 5). The interaction of carbaryl and density indicated a trend in the multivariate response, and univariate analysis showed a significant effect on de-

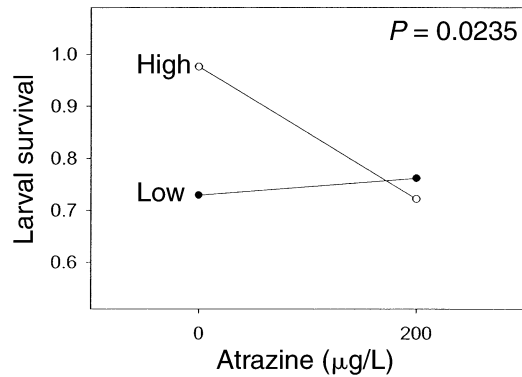


FIG. 4. Larval survival of southern leopard frog (*Rana sphenoccephala*) tadpoles in Experiment I from low- and high-density ponds across atrazine treatments.

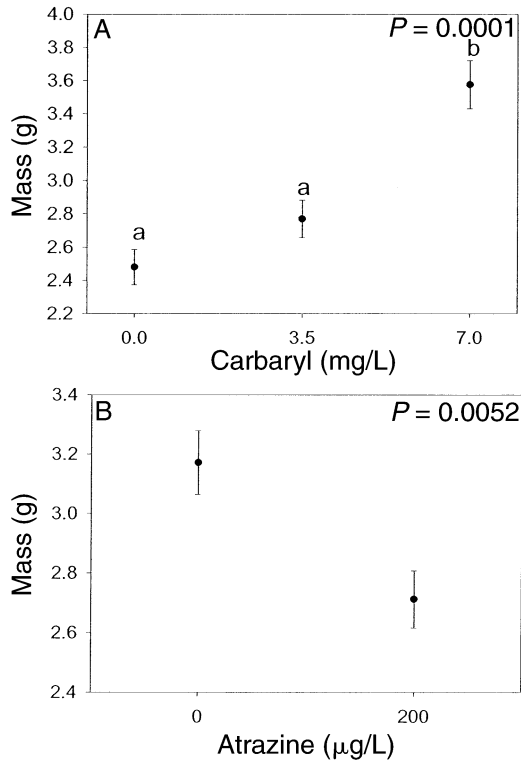


FIG. 5. Mass of southern leopard frog (*Rana sphenocphala*) tadpoles from Experiment I across (A) carbaryl and (B) atrazine treatments. Error bars represent ± 1 SE. Different letters above the data points indicate significant differences among treatments according to Scheffé's multiple comparison tests.

developmental stage (Table 1; carbaryl controls, 40.4 ± 0.5 [low density], 35.2 ± 0.5 [high density]; 3.5 mg/L, 40.4 ± 0.6 [low], 36.1 ± 0.5 [high]; 7.0 mg/L, 39.2 ± 0.6 [low], 37.6 ± 0.8 [high] Gosner stage; means ± 1 SE).

We collected a number of recently metamorphosed southern leopard frogs at the close of the experiment; however, there were not enough for statistical analysis. In low-density ponds, we collected 15 frogs in controls (containing frogs and salamanders; 1.744 ± 0.060 g), seven in competition controls (1.985 ± 0.078 g), eight exposed to atrazine only (1.431 ± 0.038 g), five exposed to 3.5 mg/L carbaryl only (2.1872 ± 0.195 g), one exposed to 7.0 mg/L carbaryl only (2.976 ± 0 g), and four exposed to atrazine and 3.5 mg/L carbaryl (1.708 ± 0.070 g); in a high-density pond, we collected one metamorph exposed to atrazine and 7.0 mg/L carbaryl (1.413 ± 0 g).

Competitive controls

Spotted salamanders reared with southern leopard frogs did not differ significantly from spotted salamanders reared alone in terms of mass ($F_{1,7} = 0.01$, $P = 0.9422$), SVL ($F_{1,7} = 0.12$, $P = 0.7433$), developmental stage ($F_{1,7} = 0.42$, $P = 0.5367$), or survival

($F_{1,11} = 0.04$, $P = 0.8374$) at the end of the experiment. Southern leopard frogs did not differ significantly in mass ($F_{1,7} = 0.36$, $P = 0.5672$), developmental stage ($F_{1,7} = 1.35$, $P = 0.2830$), or survival ($F_{1,8} = 0.30$, $P = 0.5979$) when reared alone or with spotted salamanders. However, high density significantly reduced mass ($F_{1,7} = 55.02$, $P = 0.0001$) and developmental stage ($F_{1,7} = 103.97$, $P < 0.0001$) in both single- and mixed-species ponds.

Experiment II: effects of hydroperiod, atrazine, and carbaryl on amphibians reared through metamorphosis

Small-mouthed salamander survival to metamorphosis was significantly reduced by carbaryl exposure (Fig. 1B, Table 3). Multivariate analyses on salamanders indicated that atrazine exposure, hydroperiod, and the interaction of atrazine and hydroperiod significantly affected mass and time to metamorphosis (Table 4). The atrazine multivariate effect can be attributed mainly to increasing the time to metamorphosis for salamanders (Fig. 6A, Table 3). Atrazine interacted significantly with the hydroperiod treatment (Table 4), affecting both time (Fig. 6A) and mass to metamorphosis (Fig. 6B) and resulting in longer larval periods in constant hydroperiods and smaller mass at metamorphosis in drying hydroperiods. A drying hydroperiod affected the multivariate responses mainly through reducing salamander mass at metamorphosis (Table 3; drying, 1.00 ± 0.07 g; constant, 1.31 ± 0.09 g).

Carbaryl exposure significantly reduced survival of American toads by $\sim 20\%$ (Fig. 7A). Multivariate responses of toads were significantly affected by carbaryl exposure, atrazine exposure, and a carbaryl by hydroperiod interaction (Table 4). Carbaryl exposure lengthened the larval period of toads significantly (Fig. 7B). Atrazine exposure reduced toad mass at metamorphosis significantly (Fig. 8). A significant carbaryl by hydroperiod interaction may be attributed mainly to its effect on mass with toads reared in constant hydroperiods having reduced mass with carbaryl exposure; whereas in drying ponds, toad mass increased slightly with carbaryl exposure (Fig. 9).

Chlorophyll content

The amount of chlorophyll changed significantly over time in both years (Table 5). In the first experiment, density, atrazine exposure, carbaryl exposure, and a carbaryl by atrazine interaction significantly affected chlorophyll over time (Table 5). In Experiment I, atrazine exposure lead to significantly lower chlorophyll content in pond water 12 d after exposure ($F_{1,24} = 15.65$, $P = 0.0006$; control, 3.88 ± 0.48 μg/L; 200 μg/L atrazine, 1.31 ± 0.48 μg/L), although there were no differences at the final measurement. Exposure to carbaryl also significantly reduced chlorophyll in the water 12 d after exposure ($F_{2,24} = 12.20$, $P = 0.0002$; control ponds, 5.39 ± 0.58 μg/L; 3.5 mg/L carbaryl,

TABLE 3. Summary of univariate analyses of covariance (ANCOVA) of mass, time, and survival to metamorphosis for small-mouthed salamanders (*Ambystoma texanum*) and American toads (*Bufo americanus*) from Experiment II.

Response variable	Source of variation	Sum of squares	df	F	P
Small-mouthed salamander					
Mass	Covariate (survival)	0.0009	1	0.05	0.8321
	Atrazine	0.0218	1	1.08	0.3175
	Hydroperiod	0.1839	1	9.10	0.0099
	Atrazine × hydroperiod	0.1414	1	7.00	0.0202
	Error	0.2625	13		
Time	Covariate (survival)	0.0402	1	23.93	0.0003
	Atrazine	0.0161	1	9.61	0.0084
	Hydroperiod	0.0063	1	3.75	0.0748
	Atrazine × hydroperiod	0.0156	1	9.32	0.0093
	Error	0.0218	13		
Survival	Carbaryl	9.0337	1	176.03	0.0001
	Atrazine	0.0573	1	1.12	0.3012
	Hydroperiod	0.0255	1	0.50	0.4878
	Carbaryl × hydroperiod	0.0031	1	0.06	0.8066
	Atrazine × hydroperiod	0.0061	1	0.12	0.7335
	Carbaryl × atrazine	0.1201	1	2.34	0.1392
	Carbaryl × atrazine × hydroperiod	0.0190	1	0.37	0.5491
	Error	1.2317	24		
	American toad				
Mass	Carbaryl	0.0058	1	0.35	0.5608
	Atrazine	0.1707	1	10.22	0.0040
	Carbaryl × hydroperiod	0.1017	1	6.09	0.0215
	Error	0.3842	23		
Time	Carbaryl	0.1215	1	39.93	0.0001
	Atrazine	0.0012	1	0.40	0.5321
	Carbaryl × hydroperiod	0.0001	1	0.02	0.8889
	Error	0.0700	23		
Survival	Carbaryl	0.0923	1	14.35	0.0009
	Atrazine	0.0033	1	0.51	0.4835
	Hydroperiod	0.0011	1	0.17	0.6857
	Carbaryl × hydroperiod	0.0000	1	0.00	0.9802
	Atrazine × hydroperiod	0.0180	1	2.81	0.1069
	Carbaryl × atrazine	0.0052	1	0.81	0.3766
	Carbaryl × atrazine × hydroperiod	0.0142	1	2.21	0.1503
	Error	0.1543	24		

Note: Statistics for sources of variation that were significant according to the MANCOVA for mass and time to metamorphosis are reported.

TABLE 4. Multivariate analysis of covariance (MANCOVA) for treatment effects and interactions on mass and time to metamorphosis for metamorphs of small-mouthed salamanders (*Ambystoma texanum*) and American toads (*Bufo americanus*) from Experiment II.

Source of variation	Wilks' lambda	df	F	P
Small-mouthed salamander				
Covariate (survival)	0.2945	2, 12	14.38	0.0007
Carbaryl	0.6771	2, 12	2.86	0.0964
Atrazine	0.4054	2, 12	8.80	0.0044
Hydroperiod	0.5829	2, 12	4.29	0.0392
Carbaryl × hydroperiod	0.9000	2, 12	0.67	0.5315
Atrazine × hydroperiod	0.5442	2, 12	5.02	0.0260
American toad				
Covariate (survival)	0.9548	2, 22	0.52	0.6011
Carbaryl	0.3051	2, 22	25.05	0.0001
Atrazine	0.6548	2, 22	5.80	0.0095
Hydroperiod	0.9341	2, 22	0.78	0.4724
Carbaryl × hydroperiod	0.7094	2, 22	4.51	0.0229
Atrazine × hydroperiod	0.8070	2, 22	2.63	0.0945
Carbaryl × atrazine	0.8739	2, 22	1.59	0.2271
Carbaryl × atrazine × hydroperiod	0.9493	2, 22	0.59	0.5644

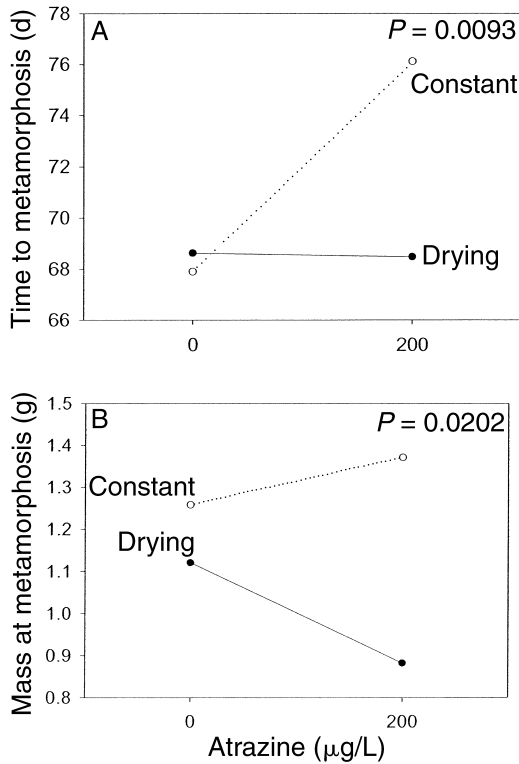


FIG. 6. Interaction of atrazine and hydroperiod on (A) time to metamorphosis and (B) mass at metamorphosis for small-mouthed salamanders (*Ambystoma texanum*) in Experiment II.

0.87 ± 0.58 µg/L; and 7.0 mg/L carbaryl, 1.51 ± 0.58 µg/L), although differences were not apparent at the final measurement. Chlorophyll was significantly affected by density over time with low-density ponds having greater amounts of chlorophyll ($F_{1,24} = 10.53$, $P = 0.0034$; low, 1.76 ± 0.43 µg/L; high, 0.53 ± 0.43 µg/L). A significant interaction between atrazine and carbaryl over time occurred 12 d ($F_{2,24} = 5.77$, $P = 0.0090$) and 25 d ($F_{2,24} = 3.98$, $P = 0.0321$) after chemical exposure (Fig. 10). At 12 d after the chemical treatment was applied, control ponds had the greatest chlorophyll levels; however, 13 d later all ponds had increased chlorophyll levels with most ponds containing more chlorophyll than control ponds.

In the second experiment, a hydroperiod by carbaryl interaction significantly affected chlorophyll over time (Table 5). However this effect was attributable to the first measurement taken before chemical addition and may result from limited chlorophyll sampling in this study ($F_{1,24} = 6.70$, $P = 0.0161$; on day 8, drying hydroperiod, 8.95 ± 2.88 µg/L [0 mg/L], 6.84 ± 2.88 µg/L [5 mg/L]; constant hydroperiod, 11.79 ± 2.88 µg/L [0 mg/L], 3.48 ± 2.88 µg/L [5 mg/L]).

DISCUSSION

Previous studies have shown that pesticides can alter amphibian communities in unexpected ways. Boone

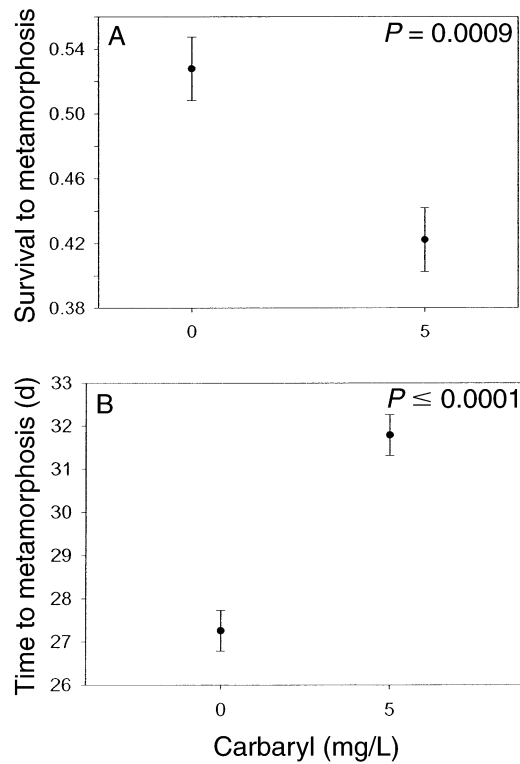


FIG. 7. (A) Survival and (B) time to metamorphosis for American toads (*Bufo americanus*) across carbaryl treatments in Experiment II. Error bars represent ± 1 SE.

and Semlitsch (2001, 2002) indicated the importance of testing contaminants in realistic conditions by incorporating natural biotic and abiotic stressors that may influence organisms' reactions to chemical stressors. In our study, natural factors of density and pond hydroperiod were also important in influencing the effects that chemicals had on amphibians. Toxicological studies should incorporate complex environments into their design because very often the difference between finding a chemical effect or not can be dependent upon

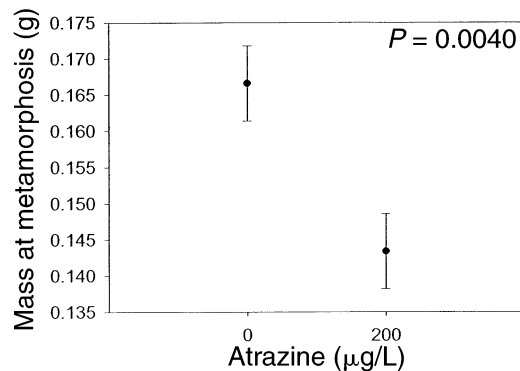


FIG. 8. Mass at metamorphosis of American toads (*Bufo americanus*) across atrazine treatments in Experiment II. Error bars represent ± 1 SE.

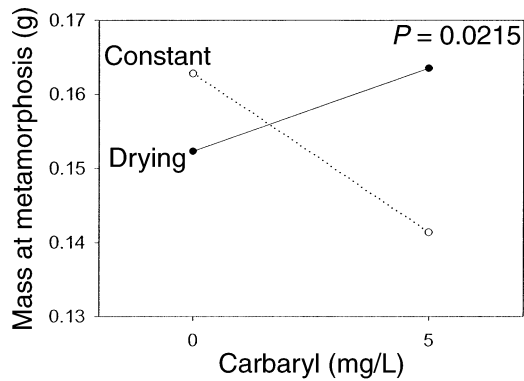


FIG. 9. Mass at metamorphosis of American toads (*Bufo americanus*) affected by a carbaryl-by-hydroperiod interaction in Experiment II.

natural factors like competition, predation, and pond hydroperiod that are known to be important in community processes (Semlitsch et al. 1996). However, in understanding the influence of contaminants in communities, it is also essential to create realistic chemical exposures, which means considering the effects of multiple contaminants and/or multiple exposures (e.g., Fairchild et al. 1994, Rowe et al. 1996, Boone et al. 2001). By using an insecticide and herbicide, we can consider the effects of two contaminants with different modes of action. Carbaryl, a neurotoxin, can alter the food web by eliminating small animals (i.e., invertebrates; Hanazato and Yasuno 1987, 1990, Mills 2002), while atrazine, a photosynthesis inhibitor, can alter the food web by reducing algal populations (Fairchild et al. 1994, 1998, Diana et al. 2000). Although direct toxicity of the tested concentrations should be low based on LC50 data (Howe et al. 1998, Boone and Bridges 1999, Bridges 1999), both of these chemicals

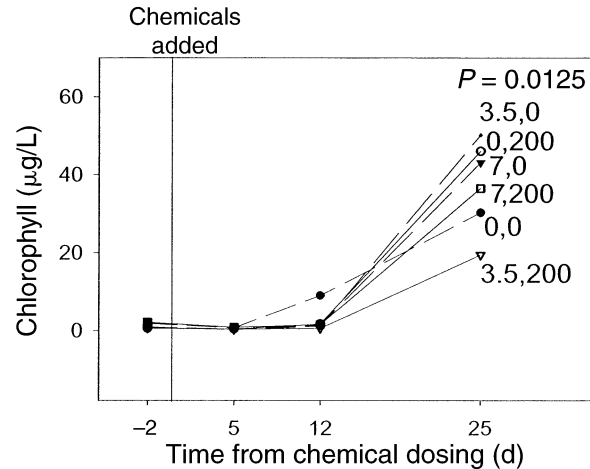


FIG. 10. Pond chlorophyll measures for an atrazine-by-carbaryl interaction over time from Experiment I. Numbers represent the concentration of carbaryl (in milligrams per liter) and atrazine (in micrograms per liter), respectively, to which the ponds were originally exposed.

have the potential to alter the food source for amphibians.

The community structure in our study was affected by contaminant exposure in ways not previously demonstrated. Both of our experiments indicated that exposure to realistic levels of carbaryl can virtually eliminate salamander larvae. Survival of the spotted and small-mouthed salamanders decreased to near zero. In contrast, toad survival dropped by ~20% while survival of the southern leopard frog was not affected by carbaryl exposure. Whether carbaryl has a positive (as in Boone and Semlitsch 2002) or negative (as in Boone and Semlitsch 2001) effect on anurans may be a function of individual, population, family, or species sen-

TABLE 5. Summary of repeated-measures ANOVA for effects of time and interactions with treatments on chlorophyll content in pond water in Experiments I and II.

Source of variation	Wilks' lambda	df	F	P
Experiment I				
Time	0.0351	3, 22	201.69	0.0001
Time × density	0.6432	3, 22	4.07	0.0193
Time × atrazine	0.6858	3, 22	3.36	0.0371
Time × carbaryl	0.5481	6, 44	2.57	0.0319
Time × density × atrazine	0.8632	3, 22	1.16	0.3466
Time × density × carbaryl	0.7334	6, 44	1.23	0.3095
Time × atrazine × carbaryl	0.4926	6, 44	3.12	0.0124
Time × density × atrazine × carbaryl	0.8057	6, 44	0.84	0.5483
Experiment II				
Time	0.1974	1, 24	97.59	0.0001
Time × hydroperiod	0.8908	1, 24	2.94	0.0992
Time × atrazine	0.9895	1, 24	0.25	0.6190
Time × carbaryl	0.9654	1, 24	0.86	0.3628
Time × hydroperiod × atrazine	0.9927	1, 24	0.18	0.6781
Time × hydroperiod × carbaryl	0.7933	1, 24	6.25	0.0196
Time × atrazine × carbaryl	0.9993	1, 24	0.02	0.8967
Time × hydroperiod × atrazine × carbaryl	0.9992	1, 24	0.02	0.8917

sitivity to contaminants (Bridges and Semlitsch 2000) or to resource availability; therefore, it is not surprising to find variation in this response among anuran species. Carnivorous salamander species, however, may have a more predictable response to insecticides if the main effect is manifested through elimination of their larval food resources (Hanazato and Yasuno 1990, Mills 2002), although our design precludes distinguishing between direct and indirect effects. Zooplankton are very sensitive to carbaryl; 48-h LC50s of carbaryl range from 6.4 to 13 $\mu\text{g/L}$ (Mayer and Ellersieck 1986). Therefore, survival of most zooplankton at levels used in our study would be unlikely. Elimination of zooplankton in the ponds following carbaryl application was observed (M. D. Boone, *personal observation*) and likely resulted in the negative impacts we found with carbaryl on growth and development for the spotted salamanders that did survive. Although carbaryl exposure can be relatively short-lived (with a half-life of $\sim 3\text{--}4$ d in our experiments), it can have a long-term effect on zooplankton (Hanazato and Yasuno 1987, Mills 2002). When zooplankton populations are reduced or eliminated, insecticides could lead to reproductive failure and subsequent population declines for carnivorous amphibian species.

In contrast, the effects of carbaryl on anurans is more difficult to predict. Carbaryl had some positive effects on mass and developmental stage of leopard frogs, as found in other studies (Boone and Semlitsch 2002). However, the survival of the American toads was reduced, and the larval period was lengthened in ponds exposed to carbaryl with constant hydroperiods, which could make individuals vulnerable to aquatic predators for longer periods of time. If an anuran community was exposed to a neurotoxin early in larval development, abundance of some species may be unaffected, increased, or reduced, and this response may depend upon the sensitivity of the anurans to direct effects of the chemical as well as the community response.

The herbicide atrazine reduced chlorophyll concentrations of algal communities (documented in Experiment I) and resulted in reduced mass (for toads and leopard frogs) and lengthened larval periods (for small-mouthed salamanders). While the presence of atrazine may not cause mortality from reductions in food resources, it may reduce metamorph size. Because size at metamorphosis has been positively correlated with overwinter survival and future reproduction (Berven and Gill 1983, Smith 1987), atrazine may affect population dynamics when it reduces metamorph size. Lengthened larval periods for salamanders may be a result of atrazine increasing energy required for growth and development (e.g., Rowe et al. 1998), although the mechanism is not clear. Furthermore, recent evidence of Hayes et al. (2002) suggests that atrazine can act as an endocrine disrupter at levels used in our study; therefore atrazine may have additional effects on surviving individuals by affecting future reproduction.

Because recent experiments evince that carbaryl can indirectly have stimulatory effects on survival and mass at metamorphosis by increasing anuran food resources (Boone et al. 2001, Boone and Semlitsch 2002, Mills 2002), we anticipated that ponds simultaneously exposed to an insecticide and herbicide would eliminate this effect for anuran species. In our study, however, there was no significant interaction of the herbicide and insecticide for any anuran species, suggesting that these chemicals may act additively. Although atrazine exposure appeared to reduce chlorophyll levels, it decreased levels to the same degree whether the ponds were exposed to carbaryl or not. That is, ponds exposed to carbaryl contained higher levels of chlorophyll than control ponds because of reduced zooplankton grazers (as in Mills 2002). If atrazine is added, the chlorophyll may be reduced proportionally so that ponds exposed to carbaryl may still have more chlorophyll than control ponds. Atrazine did interact with density and influenced leopard frog survival, suggesting that atrazine reduced the food supply of leopard frog tadpoles to some extent (as demonstrated from our chlorophyll analyses) and increased the likelihood of starvation in high-density conditions where food was scarcer.

We did find a significant carbaryl by atrazine interaction that affected larval spotted salamander SVL, mass, and developmental stage. Salamander larvae in control ponds had the largest SVL, mass, and developmental stage and the lowest in ponds that contained carbaryl only. Under carbaryl control conditions, the presence of atrazine had a negative effect on the individuals that did survive, but in the presence of carbaryl, individuals reached a greater size and developmental stage (although still lower than chemical control ponds). Why atrazine would have a positive effect on salamanders in the presence of carbaryl is unclear, but it suggests there are complex chemical or food web interactions, which warrants further investigation.

Our research suggests that there are important changes in the amphibian community that ensue from chemical exposure through altering species abundances. In both studies, we found that carbaryl reduced survival of salamanders, that atrazine had negative effects on anuran mass and time to metamorphosis, and that both chemicals interacted with other natural factors. Although both chemicals used in this study may have relatively benign direct effects on anurans and salamanders, insecticides and herbicides have a real potential to alter the food base of amphibians. Because so many pesticides are used in the environment, looking at the interactions of representative chemicals on amphibians is a useful starting point in evaluating their singular and interactive effects on these communities.

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