

## MULTIPLE STRESSORS IN AMPHIBIAN COMMUNITIES: EFFECTS OF CHEMICAL CONTAMINATION, BULLFROGS, AND FISH

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**Abstract.** A leading hypothesis of amphibian population declines is that combinations of multiple stressors contribute to declines. We examined the role that chemical contamination, competition, and predation play singly and in combination in aquatic amphibian communities. We exposed larvae of American toads (*Bufo americanus*), southern leopard frogs (*Rana sphenoccephala*), and spotted salamanders (*Ambystoma maculatum*) to overwintered bullfrog tadpoles (*R. catesbeiana*), bluegill sunfish (*Lepomis macrochirus*), the insecticide carbaryl, and ammonium nitrate fertilizer in 1000-L mesocosms. Most significantly, our study demonstrated that the presence of multiple factors reduced survival of *B. americanus* and *A. maculatum* and lengthened larval periods of *R. sphenoccephala*. The presence of bluegill had the largest impact on the community; it eliminated *B. americanus* and *A. maculatum* and reduced the abundance of *R. sphenoccephala*. Chemical contaminants had the second strongest effect on the community with the insecticide, reducing *A. maculatum* abundance by 50% and increasing the mass of anurans (frogs and toads) at metamorphosis; the fertilizer positively influenced time and mass at metamorphosis for both anurans and *A. maculatum*. Presence of overwintered bullfrogs reduced mass and increased time to metamorphosis of anurans. While both bluegill and overwintered bullfrog tadpoles had negative effects on the amphibian community, they performed better in the presence of one another and in contaminated habitats. Our results indicate that predicting deleterious combinations from single-factor effects may not be straightforward. Our research supports the hypothesis that combinations of factors can negatively impact some amphibian species and could contribute to population declines.

**Key words:** ammonium nitrate; amphibian decline; bluegill; carbaryl; chemical contamination; competition; fertilizer; insecticide; multiple stressors; overwintered bullfrogs; predation.

### INTRODUCTION

Natural communities are faced with multiple environmental factors that influence community structure (for amphibians, see Semlitsch et al. 1996; in general, see Vitousek et al. 1997). Human activities have far-reaching effects and have undoubtedly compounded the multiplicity of stressors affecting natural systems; for instance, chemical contaminants applied in one location can be redistributed globally through aerial deposition or through movement in global cycles (Bidleman 1999, van Dijk and Guicherit 1999, Thurman and Cromwell 2000). Because research demonstrates that the presence of two or more factors can cause sublethal chemical stressors to become lethal when conditions are altered (Hatch and Blaustein 2003, Relyea 2003), examining the roles of multiple stressors is essential if we are to understand how to protect community processes. Recent studies support the idea that multiple, sublethal stressors may negatively affect amphibian populations, with combinations of factors often having interactive effects

not predicted from single-factor studies (e.g., Kiesecker and Blaustein 1995, Relyea and Mills 2001, Blaustein et al. 2003, Boone and James 2003, Johnson and Sutherland 2003, Boone et al. 2005).

We examined the individual and interactive effects of two contaminants that may influence amphibian populations, the insecticide carbaryl and ammonium nitrate fertilizer, in the presence of bluegill sunfish (potential predators) and overwintered bullfrog tadpoles (potential competitors). Both carbaryl and ammonium nitrate are common in water sources due to aerial deposition and runoff (Hecnar 1995, Gilliom et al. 2006) and may even be common in protected habitats. High environmental concentrations of both contaminants can be lethal (Hecnar 1995, Bridges 2000, Hatch and Blaustein 2000), but most expected environmental concentrations appear to be sublethal (Bridges 1997, 2000, Boone and Semlitsch 2001, 2002, de Wijer et al. 2003). The effect of expected environmental concentrations of carbaryl and nitrate for amphibians appears to be mediated by changes in the food web (Boone and Semlitsch 2003, Mills and Semlitsch 2004, Boone et al. 2005, Relyea et al. 2005), which can have positive or negative effects on amphibians depending on how their food resources or predators are influenced.

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Locations where the American bullfrog (*Rana cat-esbeiana*) and bluegill sunfish (*Lepomis macrochirus*) have been introduced are associated with amphibians (e.g., Bury and Luchenback 1976, Knapp 2005). Bullfrogs have larval periods often exceeding one year and reach large sizes, allowing them to be powerful competitors in the larval environment (Kupferberg 1997, Adams 2000, Boone et al. 2004a). Bluegill and other fish can compete with larval amphibians for their resources and can reduce or eliminate many amphibians (Heyer et al. 1975, Semlitsch 1988, Knapp 2005). Further, the presence of fish facilitates overwintered bullfrog tadpoles (Werner and McPeck 1994, Adams et al. 2003, Boone and Semlitsch 2003), so the presence of both fish and bullfrogs may have stronger effects on amphibian communities than either species alone. While bullfrogs and fish are closely associated with permanent ponds, there is overlap among amphibian species across the hydroperiod gradient, the period of time during which a wetland is covered by water (Wellborn et al. 1996), which may result in amphibians and fish frequently coinciding.

We hypothesized that increasing the number of stressors present in the environment would increase the negative impacts on the populations and the community and increase the likelihood of negative synergistic interactions. We will use the term “stressors” for any factor with the potential to negatively affect the species of interest in our study; thus, all “factors” manipulated in this study are capable of being stressors by this definition. We expected negative impacts as the number of stressors increased on species diversity and abundance, as well as on mass at, time to, and survival to metamorphosis of *A. maculatum*, *R. sphenoccephala*, and *B. americanus* reared together in outdoor aquatic mesocosms.

#### METHODS

We collected 20 egg masses of *A. maculatum*, three egg masses of *R. sphenoccephala*, and seven egg strings of *B. americanus* at the University of Missouri's Basket Wildlife Area near Ashland (Boone County), Missouri, USA, on 14, 18, and 20 April 2003, respectively. Eggs of anurans were hatched in the laboratory at 23–25°C and were held until tadpoles were free-swimming (Gosner stage 25; Gosner 1960). Eggs of *A. maculatum* were placed in an outdoor pond mesocosm and collected after hatching. We mixed clutches of amphibians within species before use to homogenize genetic variation.

We established aquatic communities in 64 polyethylene ponds (1.85 m in diameter, 0.6 m in height, 1480 L volume) by adding 1000 L of water, 1 kg of leaf litter, and plankton from natural ponds (500 mL of plankton/pond) in late March at the University of Missouri Research Park in Columbia (Boone County), Missouri, USA. Screen-mesh lids covered each pond. Mesocosms are a useful and powerful way to address ecotoxicological questions at a community scale (for a review see Rowe and Dunson 1994, Boone and James 2005), and

they yield results similar to large farm ponds (Boone et al. 2004b).

We manipulated four factors in a fully crossed design with four replicates: exposure to 0 or two bluegill, 0 or six overwintered bullfrog tadpoles, 0 or 2.5 mg/L of the insecticide carbaryl, and 0 or 10 mg/L ammonium nitrate fertilizer. Bluegill were seined from a pond at the USGS Columbia Environmental Research Center; we added two bluegill to appropriate ponds after weighing them on 23 April. We collected overwintered bullfrog tadpoles from the same location in a fishless pond and added six individuals to appropriate ponds on 24 April. We added 60 *B. americanus*, 30 *R. sphenoccephala*, and 10 *A. maculatum* larvae to each pond on 25 April (experimental day 0), which is within the expected range of larval density in nature (14–4238 larvae/1000 L; e.g., Morin 1983, Petranka 1989).

We added carbaryl as liquid Sevin (22.5% carbaryl; GardenTech, Lexington, Kentucky, USA) at a nominal concentration of 2.5 mg/L (11.1 g Sevin added to 1000 L of water), below expected post-application levels in wetlands receiving direct overspray ( $\leq 4.8$  mg/L; Norris et al. 1983, Peterson et al. 1994). We added nitrate in the form of ammonium nitrate fertilizer (34:0:0, nitrogen:phosphorus:potassium) at a nominal concentration of 10 mg/L (29.4 g ammonium nitrate), which is equal to the United States' allowable drinking water level for nitrate. Environmental levels of nitrate are naturally  $< 3$  mg/L, but can reach 100 mg/L (Hecnar 1995) in waters contaminated by fertilizers. We mixed carbaryl and fertilizer with 5 L of pond water and poured the mixture evenly across the pond surface with a watering can at 13:00 hours CST on 12 May to simulate an agricultural overspray event; we added 5 L of uncontaminated pond water with a watering can to control ponds to mimic the disturbance.

Carbaryl exposure concentrations were sampled at 1, 24, 48, and 96 h following exposure. A 3-L composite sample was taken from three replicate ponds (1 L from each pond) exposed to carbaryl, from which 20 mL was removed, refrigerated, and sent to Mississippi State Chemical Laboratory (Starkville, Mississippi, USA) for analysis by HPLC. We also measured nitrate and ammonium concentrations in each pond on 12 May, 20 May, 16 June, and 7 July. We collected 60 mL of water from each pond, which was filtered and preserved with three drops of 0.5 mol/L sulfuric acid. Nitrate and ammonium concentrations were measured using a Technicon II Autoanalyzer system (Technicon Instruments, Tarrytown, New York, USA) in accordance with the manufacturer's recommendations.

We measured each pond for periphyton and zooplankton on 6 May (before dosing), 19 May, 2 June, and 7 July. Periphyton samples were taken by scraping a  $3 \times 1.3$  cm patch just below the waterline on the west side of each pond. Periphyton was wiped onto a filter 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). We took zooplankton samples by collecting composite samples from each pond, of which 1 L was filtered (80- $\mu$ m mesh) to collect zooplankton. Zooplankton were stored in 80% ethanol. We checked ponds daily for metamorphs (anurans, Gosner stage 42, Gosner 1960; salamanders, Donavan stage 55, Donavan 1980). Metamorphs were removed from each pond and held in the laboratory until tail resorption (anurans; usually  $\leq 3$  d) or measured immediately (salamanders), at which time they were towel dried and weighed to the nearest mg. Bluegill were removed on 16 July and mass was recorded. The experiment was terminated on 17 July (83 d after initiation) when it appeared that most tadpoles and salamander larvae had reached metamorphosis. We drained ponds and searched for remaining larvae or metamorphs. Larvae were not included in the analyses (differences between survival to metamorphosis and total survival did not differ substantially).

#### Response variables and statistical analyses

We examined how the number of stressors (regardless of type) influenced mass, time, and survival to metamorphosis for each species with a linear regression. We examined how species richness and evenness of *B. americanus*, *R. sphenoccephala*, and *A. maculatum* were influenced by the presence of bullfrogs, bluegill, carbaryl, nitrate, and their interactions. Evenness was calculated by Simpson's index of diversity/ $\ln(\text{species richness})$ . Simpson's index of diversity equals  $1/\sum(\text{proportion of species } i)^2$ .

Analyses for treatment effects and their interactions on mass and time to metamorphosis (referred to here as "metamorphic response") for each species were performed using MANOVA; a survival covariate was used for analyses to remove effects attributable to differences in survival among ponds. Effects of treatments and their interactions on survival to metamorphosis, species evenness, and richness were examined with ANOVA. To normalize data and stabilize variances, we angularly transformed proportion data and log transformed mass and time to metamorphosis before analyses. We measured responses of bullfrogs by examining mass, time, and survival to metamorphosis. We examined change in mass and survival of bluegill using ANOVA to test for treatment-level effects. Results for the above analyses can be found in Appendix A. Periphyton abundance, zooplankton abundance, nitrate, and ammonium levels were analyzed with a repeated-measures ANOVA to determine differences in exposure to a covariate (amphibian survival), bluegill, bullfrogs, nitrate, carbaryl, and their interactions over time.

## RESULTS

### Community patterns

Species richness significantly decreased by 28% with exposure to bluegill ( $P < 0.0001$ ; 0 bluegill/pond,  $3.000 \pm 0.046$ ; 2 bluegill/pond,  $0.844 \pm 0.046$ ; all statistics are

mean  $\pm$  SE) but was unaffected by other treatments. Evenness was significantly reduced by the presence of bluegill ( $P < 0.0001$ ) and carbaryl ( $P = 0.0003$ ) and was significantly affected by an interaction of bluegill and carbaryl ( $P = 0.0003$ ; no bluegill present, no carbaryl,  $2.064 \pm 0.022$ ; no bluegill present, 2.5mg/L carbaryl,  $1.889 \pm 0.022$ ; 2 bluegill/pond, 0 and 2.5 mg/L carbaryl, 0).

The cumulative number of stressors reduced survival of *B. americanus* (adjusted  $r^2 = 0.2125$ ,  $P < 0.0001$ ,  $n = 64$ ) and *A. maculatum* (adjusted  $r^2 = 0.3298$ ,  $P < 0.0001$ ,  $n = 64$ ) (Fig. 1a). *R. sphenoccephala* (adjusted  $r^2 = 0.0142$ ,  $P = 0.1720$ ,  $n = 64$ ) and bullfrog (adjusted  $r^2 = -0.0262$ ,  $P = 0.6501$ ,  $n = 32$ ) survival, however, were unaffected. Increasing the number of stressors present for *R. sphenoccephala* resulted in longer time to metamorphosis (adjusted  $r^2 = 0.3862$ ,  $P < 0.0001$ ,  $n = 58$ ) without an increase in mass. The bullfrogs' mass at metamorphosis was positively affected by the increasing number of factors ( $P = 0.0002$ ; Fig. 1b).

### Effects on metamorphosis

*Bufo americanus* (American toad).—Bluegill completely eliminated *B. americanus* from ponds ( $P < 0.0001$ ), but other treatments or their interactions did not affect *B. americanus* survival. The metamorphic response of *B. americanus* was significantly influenced by the presence of bullfrogs ( $P < 0.0001$ ) due to a 19% reduction in mass ( $P = 0.0111$ ) and a 2% increase in time to metamorphosis ( $P = 0.0564$ ). Nitrate exposure affected the multivariate response ( $P < 0.0001$ ) mainly through a 34% increase in mass at metamorphosis ( $P = 0.0111$ ; 0 mg/L nitrate,  $0.198 \pm 0.005$  g; 10 mg/L nitrate,  $0.266 \pm 0.005$  g). An interaction of nitrate  $\times$  carbaryl  $\times$  bullfrogs on the metamorphic response ( $P = 0.0346$ ) affected both mass ( $P < 0.0001$ ) and time to metamorphosis ( $P = 0.0210$ ; Fig. 2). With the addition of bullfrogs, *B. americanus* exposed to a single contaminant left the pond earlier and smaller, while those exposed to 0 or two contaminants left the pond later and smaller with the addition of bullfrogs.

*Rana sphenoccephala* (Southern leopard frog).—*R. sphenoccephala* was the only species (besides bullfrogs) to survive in the presence of bluegill, although survival was 59% lower than controls ( $P < 0.0001$ ; 0 bluegill/pond,  $0.8875 \pm 0.0412$ ; 2 bluegill/pond,  $0.5240 \pm 0.0412$ ). *R. sphenoccephala* survival, however, increased by 29% with carbaryl exposure ( $P = 0.0039$ ; 0 mg/L carbaryl,  $0.616 \pm 0.054$ ; 2.5 mg/L carbaryl,  $0.796 \pm 0.054$ ). The metamorphic response was positively affected by nitrate exposure ( $P < 0.0001$ ) with a 26% increase in mass ( $P < 0.0001$ ; 0 mg/L nitrate,  $1.834 \pm 0.081$  g; 10 mg/L nitrate,  $2.304 \pm 0.076$  g) and a 3% reduction in time to metamorphosis ( $P = 0.0034$ ; 0 mg/L nitrate,  $68.9 \pm 0.4$  d; 10 mg/L nitrate,  $67.0 \pm 0.4$  d) in nitrate-exposed ponds. Presence of overwintered bullfrogs affected metamorphosis of *R. sphenoccephala* ( $P < 0.0001$ ) by reducing mass by 17% ( $P = 0.0010$ ; 0

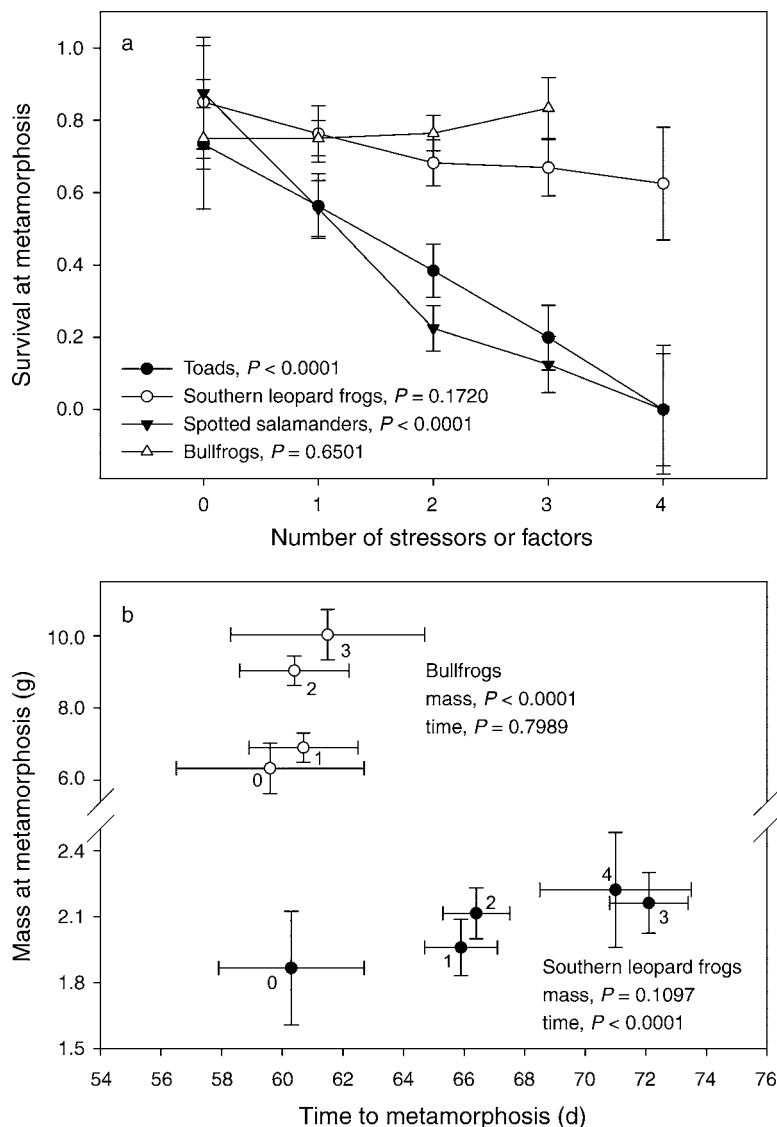


FIG. 1. (a) Effect of the number of stressors on survival to metamorphosis of amphibians and (b) on the time and mass at metamorphosis of *Rana sphenocephala* (southern leopard frogs; solid circles) and *Bufo americanus* (bullfrogs; open circles). Error bars represent  $\pm$ SE. Numbers beside points represent the number of stressors present in the aquatic environment. Data were analyzed with regression, but average values were plotted here. *P* values are taken from regression analysis and indicate the significance of the number of stressors on the response. Time stands for time to metamorphosis.

bullfrogs,  $2.267 \pm 0.076$  g; 6 bullfrogs,  $1.871 \pm 0.081$  g) and increasing time to metamorphosis by 11% ( $P < 0.0001$ ; 0 bullfrogs,  $64.1 \pm 0.4$ ; 6 bullfrogs,  $71.8 \pm 0.4$  d).

Bluegill had a significant effect on *R. sphenocephala* metamorphosis ( $P < 0.0001$ ) by increasing time to metamorphosis ( $P < 0.0001$ ; Fig. 3) without an appreciable increase in mass. The metamorphic response was also significantly affected by interactions of carbaryl  $\times$  bluegill ( $P = 0.0254$ ), bullfrogs  $\times$  bluegill ( $P = 0.0315$ ), nitrate  $\times$  bluegill  $\times$  bullfrogs ( $P = 0.0200$ ), and nitrate  $\times$  carbaryl  $\times$  bluegill  $\times$  bullfrogs ( $P = 0.0003$ ). Differences among treatments were mainly associated with differences in time to metamorphosis (nitrate  $\times$  carbaryl  $\times$

bluegill  $\times$  bullfrogs;  $P < 0.0001$ ), with *R. sphenocephala* exposed to either bullfrogs or bluegill taking longer to reach metamorphosis, especially when exposed to both bullfrogs and bluegill (Fig. 3), with no appreciable increase in mass at metamorphosis.

*Ambystoma maculatum* (Spotted salamander).—*A. maculatum* were eliminated by bluegill ( $P < 0.0001$ ). Survival was negatively affected by carbaryl exposures ( $P < 0.0001$ ) and significantly reduced by bluegill  $\times$  carbaryl treatments ( $P < 0.0001$  [0 bluegill and 0 carbaryl,  $0.825 \pm 0.036$ ; 0 bluegill and 2.5 mg/L carbaryl,  $0.4125 \pm 0.036$ ; 2 bluegill and 0 and 2.5 mg/L carbaryl, 0]). Additionally, there were trends ( $0.0500 < P <$

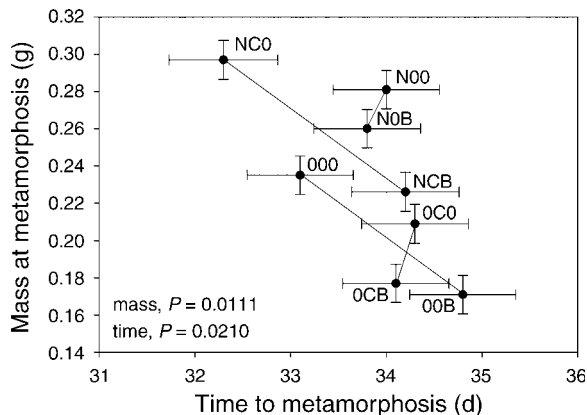


FIG. 2. Mass and time to metamorphosis of *B. americanus* affected by a significant interaction of bullfrog × carbaryl × nitrate treatments on the multivariate response of metamorphosis. Presence of nitrate, carbaryl, or overwintered bullfrog tadpoles is denoted by “N,” “C,” or “B,” respectively. Absence is indicated by a 0. Error bars represent ±SE. Lines connect chemical treatments that are exposed or not exposed to overwintered bullfrog tadpoles. *P* values are from univariate analyses for the interaction.

0.0620) for interactions with nitrate × carbaryl, nitrate × carbaryl × bluegill, and nitrate × carbaryl × bluegill × bullfrogs on survival, which indicate that the chemical environment influenced whether or not the presence of bullfrogs decreased *A. maculatum* survival (Fig. 4).

The metamorphic response of *A. maculatum* was significantly influenced by nitrate exposure ( $P = 0.0195$ ),

with a 24% increase in mass at metamorphosis ( $P = 0.0129$ ; 0 mg/L nitrate,  $1.117 \pm 0.065$  g; 10 mg/L nitrate,  $1.387 \pm 0.065$  g) and a 3% decrease in time to metamorphosis ( $P = 0.0904$ ; 0 mg/L nitrate,  $67.1 \pm 0.7$  d; 10 mg/L nitrate,  $65.2 \pm 0.7$  d) with ammonium nitrate exposure. *A. maculatum* metamorphic response was negatively affected by carbaryl exposure ( $P < 0.0001$ ), with a 39% reduction in mass ( $P = 0.0007$ ; 0 mg/L carbaryl,  $1.556 \pm 0.084$  g; 2.5 mg/L carbaryl,  $0.948 \pm 0.064$  g) and a 20% increase in time ( $P < 0.0001$ ; 0 mg/L carbaryl,  $59.9 \pm 1.0$  d; 2.5 mg/L carbaryl,  $72.4 \pm 1.0$  d) to metamorphosis. Although the effect of carbaryl × bullfrogs ( $P = 0.0830$ ) on the metamorphic response was not significant, *A. maculatum* exposed to carbaryl showed similar time to metamorphosis whether or not bullfrogs were present, while in chemical controls they left the pond 4.4 d earlier when exposed to bullfrogs ( $P = 0.0329$ ; 0 carbaryl and 0 bullfrogs,  $62.1 \pm 1.4$  d; 0 carbaryl and 5 bullfrogs,  $57.7 \pm 1.1$  d; 2.5 mg/L carbaryl and 0 bullfrogs,  $72.4 \pm 1.2$  d; 2.5 mg/L carbaryl and 5 bullfrogs,  $72.5 \pm 1.1$  d), indicating that carbaryl reduced *A. maculatum*'s ability to respond to the presence of other species.

*Effects on overwintered bullfrogs and bluegill*

Survival of overwintered bullfrogs to metamorphosis was not significantly impacted by any treatments or their interactions, although there was a moderate effect on the interaction of carbaryl × bluegill ( $P = 0.0646$ ) with greater survival to metamorphosis in the presence of both carbaryl and bluegill than in any other treatment (0 mg/L carbaryl and 0 bluegill,  $0.79 \pm 0.06$ ; 0 mg/L

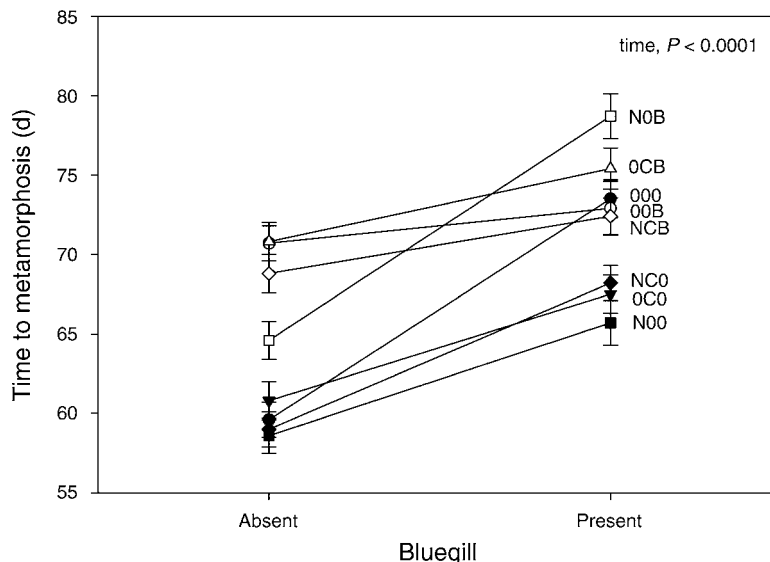


FIG. 3. Time to metamorphosis for *R. sphenoccephala* affected by a significant interaction of bluegill × bullfrog × nitrate × carbaryl treatments on the multivariate response of metamorphosis. Presence of nitrate, carbaryl, or overwintered bullfrog tadpoles is denoted by “N,” “C,” or “B,” respectively. Absence is indicated by a 0. Error bars represent ±SE. The *P* value is from the significant four-way interaction from the ANCOVA for the effect of time to metamorphosis.

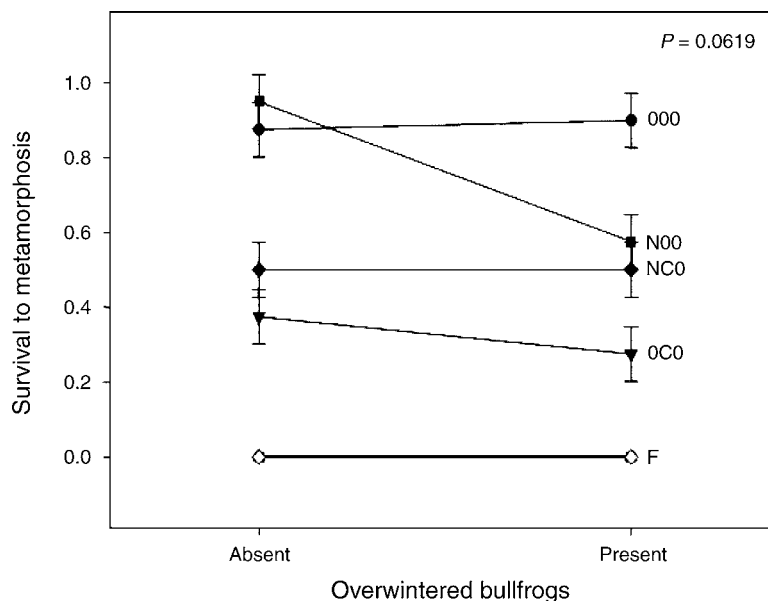


FIG. 4. Survival to metamorphosis for *Ambystoma maculatum* affected by an interaction of bluegill  $\times$  bullfrog  $\times$  nitrate  $\times$  carbaryl treatments. Presence of bluegill, nitrate, carbaryl, or overwintered bullfrog tadpoles is denoted by "F," "N," "C," or "B," respectively; "F" indicates any treatment exposed to bluegill. Absence is indicated by a 0. Error bars represent  $\pm$ SE. The  $P$  value is from ANOVA results.

carbaryl and 2 bluegill,  $0.69 \pm 0.6$ ; 2.5 mg/L carbaryl and 0 bluegill,  $0.73 \pm 0.06$ ; 2.5 mg/L carbaryl and 2 bluegill,  $0.85 \pm 0.06$ ). The metamorphic response of overwintered bullfrogs was significantly enhanced by the presence of bluegill ( $P < 0.0001$ ) through a 47% increase in mass ( $P < 0.0001$ ; 0 bluegill,  $6.498 \pm 0.220$  g; 2 bluegill,  $9.574 \pm 0.220$  g) and a 10% decrease in time to metamorphosis ( $P = 0.0113$ ; 0 bluegill,  $57.8 \pm 1.4$  d; 2 bluegill,  $63.3 \pm 1.4$  d). The effect of nitrate exposure on the metamorphic response ( $P = 0.0100$ ) could be attributed mainly to a 14% increase in mass ( $P = 0.0022$ ; 0 nitrate,  $7.504 \pm 0.220$  g; 10 mg/L nitrate,  $8.540 \pm 0.220$  g). Additionally, the metamorphic response was marginally affected by an interaction among nitrate  $\times$  carbaryl  $\times$  bluegill treatments ( $P = 0.0912$ ), which could be attributed to increased mass at metamorphosis ( $P = 0.0284$ ) in the presence of bluegill and both chemicals (Fig. 5a).

We also examined the effects of treatments on bluegill. Treatments and their interactions did not affect survival, but change in mass was influenced by an interaction among bullfrogs  $\times$  carbaryl  $\times$  nitrate treatments ( $P = 0.0264$ ), which was mainly attributed to an increase in mass of bluegill ( $P = 0.0072$ ; Fig. 5b).

#### Plankton and periphyton communities

Periphyton, the main food resource for tadpoles, increased with nitrate exposure ( $P = 0.0019$ ; Appendix B) and decreased in the presence of bullfrog tadpoles ( $P = 0.0018$ ; Appendix B) over time; there was also a short-term increase in food resources with carbaryl exposure

(time  $\times$  carbaryl,  $P = 0.0700$ ; time 2,  $P = 0.0097$ ; 0 carbaryl,  $135.6 \pm 37.5$   $\mu$ g/L of periphyton in solution; 2.5 mg/L carbaryl,  $272.6 \pm 37.5$   $\mu$ g/L of periphyton in solution) and bluegill presence (time  $\times$  bluegill,  $P = 0.1217$ ; time 2,  $P = 0.0478$ ; 0 bluegill,  $12.3 \pm 101.3$   $\mu$ g/L; 2 bluegill,  $396.0 \pm 101.3$   $\mu$ g/L). Additionally, periphyton abundance varied over time with the interaction of nitrate  $\times$  bullfrogs ( $P = 0.0010$ ), nitrate  $\times$  bluegill ( $P = 0.0032$ ), and bullfrogs  $\times$  bluegill ( $P = 0.0112$ ); while there were differences among treatments, the main effects appear to explain the general pattern.

Cladocerans were the dominant group of zooplankton in ponds and the main food resource for *A. maculatum* larvae. The abundance of cladocerans varied significantly over time ( $P < 0.0001$ ) and was reduced by the presence of carbaryl ( $P < 0.0001$ ) and the presence of bluegill ( $P = 0.0023$ ; Appendix B); reduction in cladocerans in bluegill and carbaryl treatments could, in part, explain the negative effects these treatments had on *A. maculatum*.

#### Chemical exposure

Carbaryl, with 1-Naphthol as its breakdown product, had a half-life of approximately 16 h (time since application, concentration of carbaryl [concentration of 1-Naphthol]: 1 h, 1.550 mg/L [0.825 mg/L]; 24 h, 1.086 mg/L [0.387 mg/L]; 48 h, 0.887 mg/L [0.289 mg/L]; 96 h, 0.713 mg/L [0.059 mg/L]). Ammonia levels decreased with time ( $P < 0.0001$ ), and ponds exposed to ammonium nitrate had higher levels of ammonium compared to controls at each measurement ( $P \leq 0.0002$ )

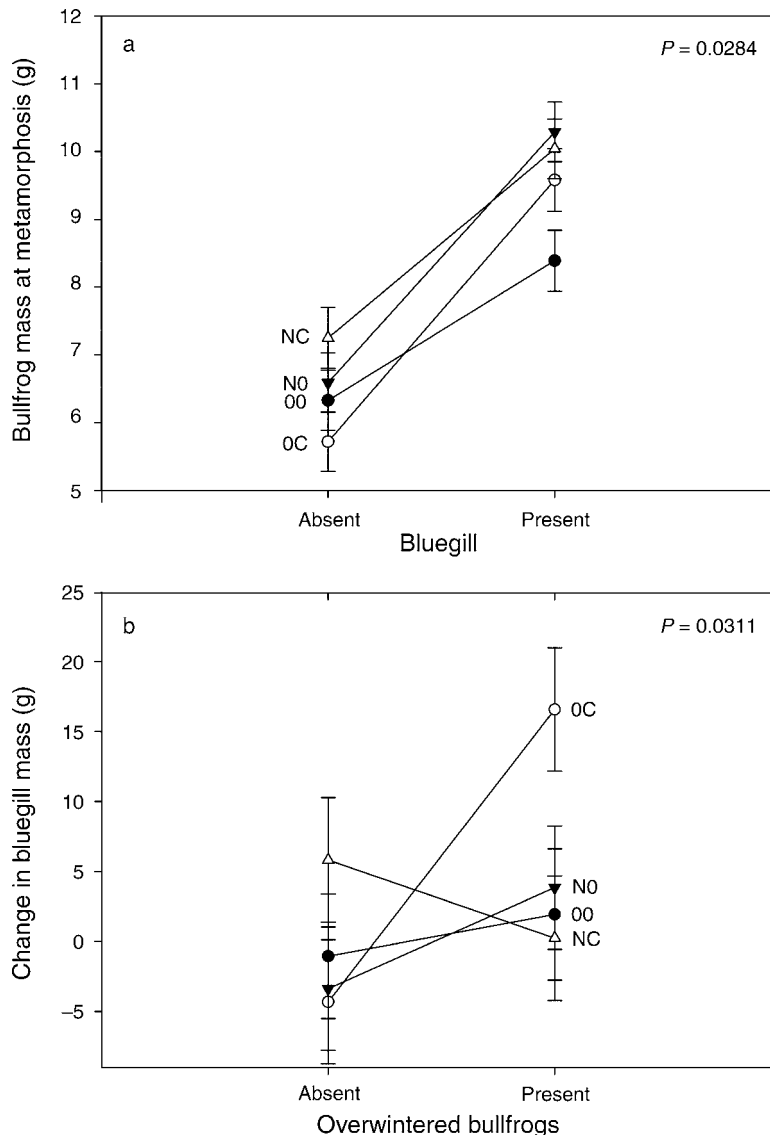


FIG. 5. Three-way interaction of treatments on (a) overwintered bullfrog’s mass at metamorphosis in the absence or presence of bluegill and (b) change in mass of bluegill during the experiment in the absence or presence of overwintered bullfrogs. Presence of nitrate and/or carbaryl is denoted by “N” or “C,” respectively. Absence is indicated by a 0. *P* values are from ANOVA results.

until the end of the experiment, when there was no significant difference between treatments (Appendix B). Nitrate levels decreased over time ( $P < 0.0001$ ) and varied with the interaction of time  $\times$  nitrate exposure ( $P = 0.0183$ ). Nitrate levels were significantly greater than controls at all, except the last, measurement dates ( $P < 0.0001$ ; Appendix B).

DISCUSSION

Our study demonstrated that combinations of stressors can impact amphibians in ways not predicted by single-factor studies alone. Many have suggested that combinations of stressors could be the underlying cause of amphibian declines (Carey et al. 2001, Linder et al.

2003, Stuart et al. 2004), and our study supports this hypothesis for some species (Fig. 1). All of the stressors impacted the amphibians with some combinations having greater than expected impacts, while other combinations ameliorated the impacts of other stressors. Here we rank the relative importance of each factor in the community, and we evaluate the potential consequences of interactions among factors to populations in nature.

*Rank order of effects*

While, in general, a greater number of stressors had negative impacts on the community, not all factors were equal. Presence of bluegill had the strongest negative effect on amphibians (excepting bullfrogs) in the study.

Bluegill completely eliminated *B. americanus* and *A. maculatum* from the ponds, which may be due to high activity in the case of *B. americanus* or poor avoidance mechanisms (Kats et al. 1988, Lawler 1989, Anholt et al. 2000). Although predation by bluegill may have been artificially high due to limited pond structure (i.e., leaf litter), bluegill are associated with reduction in amphibian abundance in natural systems (Vrendenburg 2004). Bluegill also reduced the food resources (zooplankton) of *A. maculatum*, which may have indirectly reduced *A. maculatum* survival. Additionally, bluegill as well as bullfrogs may carry pathogens like *Saprolegnia* (Kiesecker et al. 2001) and the chytrid fungus (Hanselmann et al. 2004), which could contribute to their negative effects on natural populations. However, predation by bluegill is the most likely mechanism of population reductions in our experiment. Because bluegill were associated with increases in periphyton (through reduction in cladocerans), *R. sphenocéphala* and/or bullfrogs that avoid bluegill predation may have more food resources in the presence of bluegill. Therefore, while the presence of bluegill reduced species richness and abundance, individuals that survived left the ponds at larger sizes, which is positively associated with fitness (Altwegg and Reyer 2003).

Exposure to chemical contaminants had the second largest effects on the community: carbaryl had negative effects on *A. maculatum* and positive (or no) effects on anurans, while ammonium nitrate had positive effects on all species. Overall, the presence of sublethal levels of carbaryl reduced species evenness of the ponds, mainly through a 50% reduction in the number of *A. maculatum*, which likely resulted from carbaryl toxicity to zooplankton prey (as in Boone and James 2003, Metts et al. 2005). *A. maculatum* that did survive took longer to reach metamorphosis and left at a smaller size. Because *A. maculatum* appear to complete a greater portion of their lifetime growth during the larval stage (Werner 1986, Semlitsch et al. 1988), future growth, reproduction, and survival of *A. maculatum* are likely compromised with carbaryl exposure. Carbaryl enhanced the survival of *R. sphenocéphala* (as in Boone and Semlitsch 2002, Boone and James 2003) and was likely a result of the observed increases in periphyton. Ammonium nitrate fertilizer enhanced metamorph quality of all amphibians in the community, probably because of increased periphyton resources. Although nitrate exposure can be toxic (Hecnar 1995, Hatch and Blaustein 2000), an addition of 10 mg/L ammonium nitrate benefited the amphibians for many traits. Our results and those of others indicate that the effects of nitrate and carbaryl are mediated primarily through alterations in food resources (Mills and Semlitsch 2004, Boone et al. 2005) or the predator community (Boone and Semlitsch 2003, Relyea et al. 2005); therefore, understanding which species are sensitive will help us anticipate indirect effects that may cascade up and down the food web.

Presence of overwintered bullfrog tadpoles, which compete with other anurans for periphyton, negatively affected some of the amphibians in the community. All amphibians in our study survived equally well with bullfrogs as without, but bullfrog presence reduced mass and increased time to metamorphosis for the other anurans. The reduction in larval fitness may also reduce survival and reproductive success in the terrestrial environment (e.g., ranids; Berven 1990, Altwegg and Reyer 2003), which would negatively impact population dynamics. Although there is some evidence that size at metamorphosis of some species may be compensated for in the terrestrial environment (in the lab, Goater 1994; in the field, Boone 2005), there may be other costs to smaller terrestrial size, like greater vulnerability to predators and increased rate of desiccation (Wilbur and Collins 1973, Werner 1986). Further, in our study *B. americanus* that remained in the ponds for longer periods of time with bullfrogs did not benefit from increased size at metamorphosis (Fig. 2). Extending the time to metamorphosis even for two days for species like *B. americanus*, which frequently breed in very ephemeral environments, could reduce metamorphic success and juvenile recruitment. However, when they are developing with bullfrogs in more permanent ponds, this is unlikely to be the case. While bullfrogs did not affect species richness or evenness, they reduced the quality for other anuran metamorphs, so bullfrogs could negatively impact population dynamics indirectly.

Bluegill and overwintered bullfrog tadpoles were mutually beneficial to one another. Other studies have found positive effects of bluegill reared with bullfrogs, which has been attributed to bluegill removing aquatic insect predators (Werner and McPeck 1994) and increasing algal food resources (Boone and Semlitsch 2003). While bullfrogs reached larger sizes at metamorphosis in the presence of bluegill, the addition of the chemical stressors bolstered this effect. Additionally, bluegill gained more mass during the course of the experiment when reared with bullfrogs in most treatments. Explaining the positive effect of bullfrogs on bluegill is less clear, but could be related to nutrient cycling (Seale 1980, Beard et al. 2002). Because bullfrogs and bluegill positively influence one another and negatively affect some amphibians in terms of survival, mass, and time to metamorphosis, the amphibian community exposed to both species is in the greatest jeopardy. Removing bluegill from ponds that were historically fishless (as in Vrendenburg 2004) would reduce the success of bullfrogs and reduce their impact on the rest of the amphibian community.

#### *Interactive effects*

The most interesting question our study addressed was how combinations of stressors influence an amphibian community. The effects observed for single factors (Table 1) could be used to make predictions about combinations of factors. While each factor alone

TABLE 1. Summary of statistically significant factors on amphibian metamorphosis, bluegill, and plankton communities.

Metric	Carbaryl	Nitrate	Bluegill	Bullfrogs
<i>Bufo americanus</i>				
Survival			-	
Mass		+		-
Time to metamorphosis				-
<i>Rana sphenoccephala</i>				
Survival	+		-	
Mass		+		-
Time to metamorphosis		+	-	-
<i>Ambystoma maculatum</i>				
Survival	-		-	
Mass	-	+		
Time to metamorphosis	-	+		
Overwintered bullfrogs				
Survival				
Mass		+	+	
Time to metamorphosis			+	
Bluegill sunfish				
Survival	+			
Change in mass	-			
Zooplankton				
Abundance	-		-	
Periphyton				
Abundance	+	+		-

Note: Plus and minus signs indicate predicted positive or negative effects on fitness based on significant effects on responses at metamorphosis. Empty cells indicate no statistically significant effect.

impacted the community, combinations were not always predictable. For instance, *B. americanus* mass to metamorphosis was enhanced by nitrate exposure, reduced by competition with overwintered bullfrog tadpoles, and unaffected by carbaryl exposure (Table 1). We would predict, therefore, that effects from both nitrate and overwintered bullfrogs may roughly balance out, which is supported by the data (Fig. 2). We would not predict a stronger positive effect of both carbaryl and nitrate presence based on single factor effects or that the addition of bullfrogs would result in a disproportionately negative effect compared to single chemical treatment (Fig. 2). Similarly, time to metamorphosis for *R. sphenoccephala* exposed to neither bullfrogs nor bluegill reached metamorphosis more quickly, while those exposed to both had longer larval periods (Fig. 3). The magnitude of the effect of bluegill or bullfrog tadpoles varied with chemical treatment, again with some chemical treatments resulting in longer delays in metamorphosis without gains in mass. Further, the effect of the overwintered bullfrogs on *A. maculatum* survival depended on the chemical environment (Fig. 4), suggesting that *A. maculatum* may be best able to deal with additional biotic stress in the absence of chemicals. These results suggest the greater likelihood of nonadditive effects with the addition of other factors, which makes the presence of anthropogenic contaminants of particular concern for evaluating causes of amphibian declines.

Negative synergistic interactions between carbaryl and ammonium nitrate were not observed as predicted; in fact, both *R. sphenoccephala* and *B. americanus* reached the greatest masses with relatively short larval period when exposed to both contaminants. In contrast, Boone et al. (2005) found that the presence of both carbaryl and nitrate negatively impacted *R. clamitans* tadpoles, while each chemical alone had positive effects on the tadpoles; therefore, impacts will likely differ among species. Interestingly, while both chemicals had positive effects for anurans and nitrate had positive effects on *A. maculatum* in our study, positive chemical effects disappeared and often resulted in a negative outcome for survival, size, or time to metamorphosis in combination with other factors. Likewise, Kiesecker (2002) found that the presence of sublethal contaminants increased susceptibility to trematode infection and also reduced the immune response. Therefore, sublethal contaminants may increase susceptibility to other factors present in the environment.

The effect of a stressor on a community is unlikely to act alone, and the outcome may depend largely on its interactions with other factors (Boone and Semlitsch 2001, 2002, 2003, Relyea 2005, Rohr and Crumrine 2005). Further, in some cases, a stressor will have a negative effect on some aspect of the community, while having a positive effect on others. For instance, while predation has obvious negative effects on abundance in amphibian populations, the presence of predators can ameliorate the effect of competition by decreasing the number of competitors and result in a greater number of individuals of competitively inferior species (e.g., Morin 1983). Likewise, contaminants can change the food web in ways so that some organisms benefit, while others may not. Consequently, our approach may need to change from a focus on negative anthropogenic effects, per se, to examining how a community will deviate from its natural structure or trajectory, so that we may evaluate how these factors may be influenced by other potential stressors in the environment.

Pond-breeding amphibians evolved in ecosystems where they deal with "multiple stressors" like competition, predation, and pond drying (Wilbur 1987, Semlitsch et al. 1996), which influence juvenile recruitment or failure in a given year. As a result, amphibians and other taxonomic groups with complex life histories may deal well with combinations of stressors during larval development. For instance, in the presence of two sublethal factors, there are few instances of species experiencing complete reproductive failure (although some laboratory studies suggest that this is possible [e.g., Relyea and Mills 2001, Relyea 2004]). Therefore, testing a multiple stressors hypothesis requires examining a large number of factors and incorporating factors known to regulate communities into designs to assess how communities, species richness, and species diversity are affected. Our results suggest that the presence of contaminants in an environment, which when evaluated

alone may have positive or no effects, can have negative consequence for species in the presence of other stressors.

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#### APPENDIX A

Multivariate and univariate analyses examining treatments and their interactions for *R. sphenoccephala*, *B. americanus*, *A. maculatum*, bullfrogs, bluegill, species richness, and evenness of the community (*Ecological Archives* A017-011-A1).

#### APPENDIX B

Figures illustrating significant effects on anuran and caudate food resources and ammonium nitrate degradation in the experiment (*Ecological Archives* A017-011-A2).