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MULTIPLE STRESSORS AND SALAMANDERS: EFFECTS OF AN HERBICIDE, FOOD LIMITATION, AND HYDROPERIOD

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Abstract. Amphibian populations can be affected adversely by multiple biotic and abiotic stressors that together can contribute to their local and global decline. We focused on the combined effects of food limitation, drying conditions, and exposure to possibly the most abundant and widely used herbicide in the world, atrazine. We used a factorial design to evaluate the effects of exposure to four ecologically relevant doses of atrazine (approximate measured doses: 0, 4, 40, and 400 μg/L), two food levels (limited and unlimited food), and two hydroperiods (presence or absence of a dry down) on the survival, life history, and behavior of the streamside salamander, *Ambystoma barbouri*, from the embryo stage through metamorphosis. In general, food and atrazine levels did not interact statistically, and atrazine affected dependent variables in a standard, dose-dependent manner. Exposure to 400 μg/L of atrazine decreased embryo survival and increased time to hatching. Drying conditions and food limitation decreased larval survival, while 400 μg/L of atrazine only reduced larval survival in one of the two years tested, suggesting that the lethality of atrazine may be condition dependent. Sublethal effects included elevated activity and reduced shelter use associated with increasing concentrations of atrazine and food limitation. The larval period was lengthened by food limitation and shortened by 400 μg/L of atrazine. Drying conditions accelerated metamorphosis for larvae exposed to 0 and 4 μg/L of atrazine but did not affect timing of metamorphosis for larvae exposed to 40 or 400 μg/L of atrazine. Food limitation, drying conditions, and 400 μg/L of atrazine reduced size at metamorphosis without affecting body condition (relationship between mass and length), even though feeding rates did not differ significantly among atrazine concentrations at any time during development. This suggests that high atrazine levels may have increased larval energy expenditures. Because smaller size at metamorphosis can lower terrestrial survival and lifetime reproduction, resource limitations, drying conditions, and environmentally realistic concentrations of atrazine have the potential to contribute to amphibian declines in impacted systems.

Key words: Ambystoma barbouri; *amphibian decline; atrazine; behavior; embryos; larvae; life history; metamorphosis; ontogeny; survival.*

INTRODUCTION

The global decline of amphibians is currently an international crisis (Wake 1991, Blaustein and Kiesecker 2002). Some of the interest in amphibian declines is due to the presumed value of amphibians as bioindicators of environmental stress, and thus harbingers of possible risk to humans. One of the few established generalizations regarding amphibian declines is that they can be caused by the complex interactions of multiple stressors (Blaustein and Kiesecker 2002). Traditionally, competition caused by food limitation and drying water bodies has been a focal stressor in amphibian life-history studies (e.g., Wilbur 1977, Semlitsch and Caldwell 1982, Semlitsch and Reyer 1992, Scott 1994). More recently, pesticide exposure has become an amphibian stressor that has received substantial scientific attention (e.g., Boone and Semlitsch 2002, Hayes et al. 2002*a*, *b*, Boone and James 2003, Carr et al. 2003). While the consequences of density-dependent food limitation and changes in pond hydroperiod on amphibian population dynamics are well established, the impacts of pesticides at ecologically relevant concentrations remain unclear. More interestingly, the combined effects of food limitation, drying conditions, and pesticide exposure on amphibians are even less well understood.

Amphibians may be particularly valuable in evaluating environmental stress from pesticides because they have permeable skin and eggs that readily absorb chemicals from the environment. Many species complete their life cycles in ponds and streams adjacent to agricultural fields where pesticides are applied, and these applications often coincide with the development of the vulnerable embryo and larval life-history stages. Moreover, pesticides may accumulate and concentrate

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both in amphibians themselves and in the water bodies they occupy (Streit 1992, Allran and Karasov 2000), and the amphibious life-style of many species exposes them to both terrestrial and aquatic contaminants. As a result, agricultural sites where pesticides are often used can have lower amphibian species richness and abundance than adjacent nonagricultural sites (Bonin et al. 1997), in some cases resulting in the disappearance of amphibians from agricultural landscapes (Berger 1989).

The herbicide atrazine is of particular interest to understanding possible causes of amphibian declines because of its widespread and abundant use. Atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) is an inhibitor of photosynthesis that is used predominantly for weed control in corn and sorghum production. It is the most commonly applied herbicide in the United States and possibly the world, with use in over 80 countries (Solomon et al. 1996). Furthermore, atrazine can be quite persistent, with estimated half-lives in aquatic environments often exceeding 100 days (Diana et al. 2000), and can bioconcentrate in amphibian larvae (Allran and Karasov 2000). Despite its prevalence and persistence, few studies have examined lethal or sublethal effects of atrazine at ecologically relevant doses. Most recently, Hayes et al. (2002*b*) found that the endocrine-disrupting effects of atrazine concentrations as low as 1 μg/L, one-third of the allowable amount in drinking water, produced hermaphroditic frogs. This underscores the need to better understand the potential contribution of this herbicide to the global and regional losses of amphibians.

Atrazine, food limitation, and habitat ephemerality have all been shown to reduce growth rates and size at metamorphosis (e.g., Wilbur 1977, Semlitsch and Caldwell 1982, Travis 1984, Larson et al. 1998, Diana et al. 2000), and thus the combination of these factors may pose greater risk to amphibians than each threat alone. Consequently, we used a factorial design to examine the effects of atrazine exposure (0, 4, 40, or 400 μg/L), food abundance (low vs. high), and hydroperiod (constant vs. lowered water level) on the survival, life history, and behavior of streamside salamanders, *Ambystoma barbouri*, from the embryo stage through metamorphosis.

There were several reasons to focus on the streamside salamander. First, the effects of contaminants on amphibians are grossly understudied (fewer than 3% of vertebrate toxicity studies between 1972 and 1998; Sparling et al. 2000) and heavily biased toward experiments on anurans (frogs and toads), with few conducted on caudates (salamanders). Second, streamside salamanders likely experience frequent and simultaneous hunger-, desiccation-, and atrazine-related stress. *A. barbouri* is found in ephemeral streams that run through the agricultural landscape of central Kentucky, southern Indiana, and southern Ohio (Petranka 1998), a region of frequent atrazine use (Solomon et al. 1996). In this region, eggs are laid under submerged rocks from January to April, hatching is from March to May, and larvae metamorphose from May to July (Petranka 1998). Consequently, egg and larval development coincide with the heaviest periods of pesticide application on neighboring cropland (Solomon et al. 1996). Larvae are generally restricted to the upper portion of streams by heavy fish predation downstream (Sih et al. 1992), which forces them to develop in high densities where competition for food can be intense (Petranka and Sih 1986). This competition is further intensified by the need to develop rapidly and metamorphose before streams dry (Petranka and Sih 1987). The final reason for focusing on *A. babrouri* is that they are of conservation concern (although it is not known if *A. barbouri* are in decline), and thus might provide insight into declines of threatened or endangered amphibian species. The restricted range of *A. barbouri* consists of a relatively small number of populations (Petranka 1998), many of which have very little gene flow with other populations (Storfer 1999). Because of these metapopulation dynamics, many populations may be incapable of compensating for local declines (Marsh and Trenham 2001) and may contain little variation on which selection can act. Also, data suggest that species with limited ranges may be narrowly adapted, and thus more susceptible to contaminants (Fioramonti et al. 1997).

Methods and Materials

Animal collection and maintenance

Thirty-eight *A. barbouri* egg clutches were collected from Fossil Creek (Jessamine County, Kentuky, USA) in late February 2002 and 2003. This was a small portion of the total number of egg masses present there and was thus unlikely to influence local population dynamics. The eggs were separated (Harrison stage 18–28), placed into an aquarium, and mixed thoroughly to randomize genetic variation. Thirty-one eggs in 2002 and 40 eggs in 2003 were arbitrarily placed into each of 48 aquaria (37 L) located in a room maintaining a 12-h light : dark photoperiod. The aquaria, wrapped in black plastic and placed on shelving covered with white paper, contained 9.5 L of constantly aerated, charcoal-filtered, dechlorinated municipal water (pH ~8, 15°C). In addition, each aquarium contained a green, translucent, glass refuge plate (26.8 × 24.3 cm) that covered half the aquarium floor. The refuge plate was hung at an angle from the top of the aquarium (with monofilament line) so that it was 1.5 cm from the bottom at the aquarium center and 11 cm from the bottom at the aquarium wall. A 3 × 24.3 cm strip of the refuge was above the water line, allowing individuals to crawl out the water after metamorphosis.

Dosing and water changes

In 2002, a stock solution of atrazine was prepared by dissolving 1.27 mg of technical grade atrazine (ICN

Laboratories, Aurora, Ohio, USA; purity = 80%, 20% inert compounds) per mL of dimethyl sulfoxide (DMSO), and was stored in an amber glass bottle at −20°C. Twelve randomly selected aquaria were treated with either 3.75 mL of DMSO, or 37.5 μL, 375 μL, or 3.75 mL of atrazine stock solution, producing nominal concentrations of 0, 4, 40, and 400 μg/L of atrazine. The same nominal concentrations were used in 2003, but a separate stock solution was prepared: 8.984 mg of atrazine (Chemservice, West Chester, Pennsylvania, USA; purity = 99%) was dissolved per milliliter of acetone and aquaria were treated with either 423 μL of acetone, 423 μL, or 42.3 μL of stock solution, or 42.3 μL of a 10-fold serial dilution of the stock solution.

Aquaria were first treated with these atrazine concentrations on the day that eggs were distributed (4 April in 2002 and 19 March in 2003), referred to as experimental day 1. *A. barbouri* were exposed to these concentrations until metamorphosis. Chronic exposure to atrazine may be common for amphibians that inhabit ponds because the reported half-life of atrazine in freshwater often exceeds 100 days (Diana et al. 2000). We did not include a water control in our design because similar solvent concentrations were shown to have no significant effects on *A. barbouri* survival, life history, or behavior (Rohr et al. 2003). Flame ionization detection (FID) gas chromatography was used to verify actual concentrations of stock solutions each year and to measure actual atrazine concentrations in each aquarium, before and after water changes, at various points during the 2002 experiment (A. Elskus, *unpublished data*).

Our atrazine concentrations were selected using a range-finding procedure. The two highest concentrations represent potential levels from run-off events near application sites and the concentration of atrazine as streams dry (Kolpin et al. 1997, Battaglin et al. 2000), while the lower concentration represents a slightly higher level of atrazine than allowed in drinking water (EPA 2002). We believe our atrazine concentrations are ecologically relevant because all concentrations may be encountered by *A. barbouri* and other amphibians in the wild. Atrazine levels in water adjacent to treated fields have been reported at 500 μg/L (de Noyelles et al. 1982) and 1000 μg/L (Kadoum and Mock 1978). In the Midwestern United States, atrazine can be up to 40 μg/L in precipitation, 224 μg/L in streams, and 2300 μg/L in tailwater pits of agricultural areas (Nations and Hallberg 1992, Kolpin et al. 1997, Battaglin et al. 2000). Although we do not have data from our collection site documenting the extent of *A. barbouri* exposure to atrazine, an atrazine concentration of 33.9 μg/L has been detected in a Kentucky stream similar in size to the stream from which we collected *A. barbouri* for this study (K. Schaffer, *personal communication*).

Full water changes were conducted every week. Water temperature, dissolved oxygen (DO), and pH were measured in each aquarium before water changes on experimental days 14, 28, 42, and 56 of 2002 only to determine if levels of these parameters posed risk to larvae. Temperature was measured with a digital thermometer (±0.1°C), and DO and pH were measured with meters (ICM Model 31200, ±0.01 mg/L [Industrial and Chemical Measurement Company, Hillsboro, Oregon, USA], and Denver Model 215, ±0.002 [Denver Instruments Company, Denver, Colorado, USA]; respectively).

Feeding regime and rates

A factorial design was used to completely cross the four atrazine concentrations with two feeding regimes (4 × 2 design with six replicates of each treatment combination). Larvae in half the aquaria of each atrazine concentration were fed live blackworms, *Lumbriculus variegatus*, ad libitum, while larvae in the other half were rationed 2.24 g (±0.005 g) twice a week after any remaining blackworms were removed. Larvae fed ad libitum are referred to as ''high-food'' larvae, and those fed rations are referred to as ''low-food'' larvae. This feeding regime represented an adequate food supply early in development, but yielded a 4–7-fold difference in food abundance later in development (J. Rohr, *unpublished data*). Feeding rates were assessed for high-food larvae on experimental days 27, 41, and 55 by placing the same mass of blackworms in each aquarium and subtracting the weight of the blackworms remaining after 24 h.

Dry down

In 2003 only, we crossed the atrazine and food treatments with two hydroperiods (constant or lowered water level) resulting in 4 × 2 × 2 design with three replicates of each treatment. However, the reduction in water level did not begin until experimental day 45 (when many larvae began substantial gill absorption) because natural stream drying does not typically occur until later in *A. barbouri* development. Water level was reduced by removing 1 L of water from the appropriate aquaria every other day until these aquaria reached 2.5 L of water. This volume was then kept constant for the rest of the experiment (8-cm to 2-cm depth reduction). Dosing procedures were adjusted so that atrazine concentration remained unchanged in dry-down aquaria.

Behavior, life history, and survival

Every other day, we counted the number of embryos and larvae in each aquaria, removed dead embryos and larvae, and removed and measured new metamorphs (wet mass, snout–vent length [SVL]), defined as those animals with fully absorbed gills. On experimental day 16, after 99% of the embryos had hatched, larvae were arbitrarily removed to ensure that all aquaria had 24 larvae. This was done to reduce the starting variation

in the number of larvae between aquaria and to obtain tissue samples to begin a study assessing the effects of atrazine on the endocrinology of *A. barbouri* (B. Shepherd, *unpublished data*). Once each week at 0900, 1200, and 1500 hours EST from experimental days 12 to 54, the number of larvae under refuge and those moving over a 15-s period were recorded.

Hypotheses

Through possible toxic effects, atrazine was expected to reduce survival, growth, and feeding, and alter behavior. We expected a multiplicative reduction in survival when exposed to both contaminants and resource restrictions because this result is most common in the literature (e.g., Kluttgen and Ratte 1994, Bridges et al. 1997, Hopkins et al. 2002). Evidence also suggests that pesticide exposure can cause lethargy, which inhibits standard behavioral responses to hunger (Rohr et al. 2003). Consequently, we hypothesized that increasing concentrations of atrazine would induce greater lethargy, which in turn would reduce the percentage of larvae out of refuge and active when hungry (atrazine-by-food interaction). Atrazine can elevate thyroxine levels that stimulate metamorphosis in salamanders (Larson et al. 1998), and thus we predicted that atrazine would stimulate early metamorphosis at a small size. Atrazine was expected to interact statistically with hydroperiod (e.g., Boone and James 2003) resulting in a synergistic reduction in larval survival and time to, and size at, metamorphosis.

Statistical analyses

All statistical analyses were conducted using Statistica 5.5a (Statsoft, Incorporated, Tulsa, Oklahoma, USA). Data presented as percentages were arcsine square-root transformed to reduce heteroscedasticity and distribution skewness. The main effect of year was significant for every dependent variable, and was thus included as a blocking variable in analyses of variance if it did not interact significantly with any treatments. Because some response variables were correlated, we conducted three separate multivariate analyses of variance (MANOVA) to analyze our hatching (mean hatching day and percentage hatched), metamorphosis (mean day of, and SVL at, metamorphosis), and water quality data (temperature, DO, and pH), and then followed these tests with univariate analysis of variance (ANOVA). We used one-way ANOVAs to evaluate the effects of the four atrazine concentrations on mean hatching day, percentage hatched, and percentage of *A. barbouri* surviving until day 16. We conducted two-way ANOVAs to examine the effects of atrazine concentration and food abundance on larval behavior (mean percentage in refuge and moving), percentage surviving after day 16, metamorphosis day, and metamorph mass and length. The effect of atrazine on the combined embryo and larval life-history stages could not be accurately measured because of the removal of larvae from each aquarium on day 16. Repeated-measures ANOVAs were used to test the effects of treatment on water quality (temperature, DO, and pH) and larval feeding and growth rates through time. A three-way ANOVA was used to examine the effects of atrazine, food level, and hydroperiod on percentage of larval survival (repeated-measures using percentage survival before and during dry down) and time to, and size at, metamorphosis. Adding a dry down in 2003 provided only three replicates for each treatment combination, and thus to increase statistical power, we pooled our two lowest and two highest atrazine concentrations. We regressed the log of mean metamorph mass (dependent variable) against the log of mean metamorph SVL within each year and used these residuals in ANOVAs to determine how year, food, hydroperiod, and atrazine affected body condition. U-shaped dose curves have been reported for salamander responses to atrazine (Larson et al. 1998), making predictions about the relationship between dose and response difficult. Consequently, where the main effect of atrazine was significant ($P < 0.05$), we examined all pairwise comparisons among concentrations using a Tukey's honestly significant difference multiple comparison test (HSD).

RESULTS

Effects on embryos and recently hatched larvae

MANOVA revealed that atrazine significantly affected hatching variables (Wilks' $F_{6, 180} = 9.80$, $P < 0.001$). Atrazine influenced mean hatching day (Wilks' $F_{3,91} = 8.05$, $P < 0.001$), percentage of embryos hatching (Wilks' $F_{3,91} = 13.11$, $P < 0.001$), and percentage of *A. barbouri* surviving until day 16 (Wilks' $F_{3,91} = 11.60$, $P < 0.001$). However, only embryos exposed to 400 μg/L of atrazine hatched later and were less likely to hatch and survive to day 16 than control larvae (HSD, $P < 0.001$; Fig. 1 and Table 1). Embryos in 40 and 400 μg/L of atrazine vigorously moved inside the eggs, but appeared to have greater difficulty breaking through the egg's jelly coating. Most embryo mortality was associated with a white film covering the embryo, suggesting the presence of a fungal pathogen, though whether the fungi caused or simply followed mortality is unknown.

Effects on larval survival

In aquaria that were not dried down, larval survival after day 16 was lower in low-food than high-food aquaria ($F_{1,56} = 15.09$, $P < 0.001$) and lower in 2003 than 2002 ($F_{1,56} = 18.68$, $P < 0.001$), but atrazine did not interact significantly with food abundance ($F_{3,56} = 1.04$, $P = 0.381$; Fig. 2). The lower survival in 2003 was primarily due to atrazine inducing significantly greater mortality in this year (Atrazine × year: $F_{3,56} = 5.26$, $P = 0.003$; Fig. 2). In 2002, there was no significant effect of atrazine ($F_{3,40} = 1.26$, $P = 0.303$),

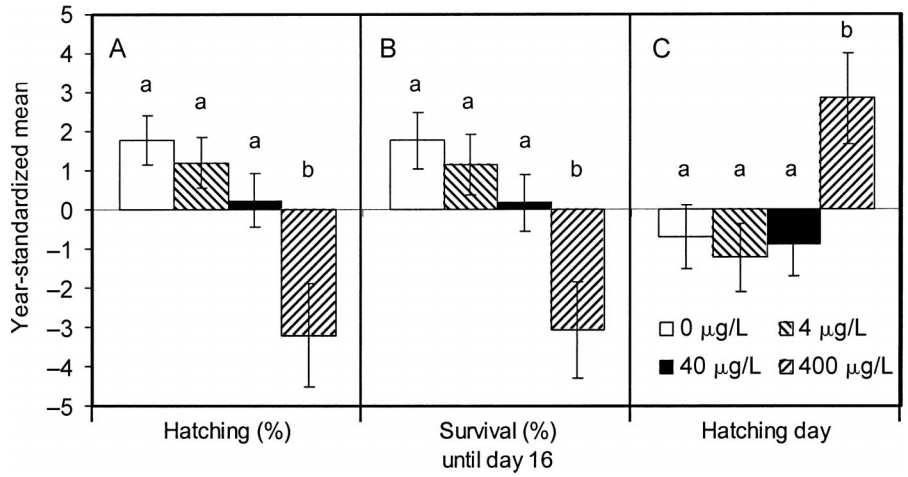

FIG. 1. Effects of atrazine (0, 4, 40, 400 μg/L) on (A) percentage of embryos hatching, (B) percentage of *Ambystoma barbouri* surviving until day 16, and (C) hatching day. There were no significant atrazine- or food-by-year interactions, so data were standardized using *z* scores (mean = 0, SD = 1) to account for differences between the two experimental years. Different lowercase letters above bars reflect significant differences among treatments according to Tukey's honestly significant difference multiple comparison tests. Bars represent means (±1 SE) of 24 replicate aquaria (12 per year). See Table 1 for actual mean values.

but in 2003, larvae exposed to 400 μg/L of atrazine had significantly lower survival than control larvae ($F_{3,16} = 4.08$, $P = 0.025$; HSD, $P = 0.019$, other $P > 0.137$).

Before the dry down began in 2003 (days 16–45), there were no significant differences in percentage survival between the larvae in the low- or high-atrazine aquaria (mean ± 1 SE: 0 and 4 μg/L, 84.53 ± 3.02%; 40 and 400 μg/L, 84.20 ± 2.22%; $F_{1,40} = 0.04$, $P = 0.845$) or between larvae in the dry-down and constant water-level aquaria (mean ± 1 SE: 82.28 ± 2.28%, 86.46 ± 2.91%, respectively; $F_{1,40} = 2.68$, $P = 0.110$). During the dry-down period (day 46 on), the reduction in water level significantly reduced survival for larvae exposed to 0 and 4 μg/L of atrazine, but had no effect on survival for the pooled group exposed to 40 or 400 μg/L of atrazine (Atrazine × dry down: $F_{1,40} = 4.77$, $P = 0.035$; Fig. 3A). Food-by-dry down and atrazine-by-food interactions were not significant ($P > 0.06$).

Effects on larval activity and refuge use

Refuge use declined precipitously for low-food larvae after day 26, which corresponded with the full consumption of allotted blackworms, suggesting that this change in behavior was consistent with the onset of food limitation and hunger. Consequently, behavioral analyses were limited to observations between days 26 and 47 (four total), the latter of which was the last observation before substantial drying occurred in 2003. Refuge use was significantly lower in 2003 than 2002 (mean ± 1 SE: 73.36 ± 1.42%, 76.70 ± 1.38%, respectively; $F_{1,80} = 4.81$, $P = 0.031$), in low-food than high-food groups (69.16 ± 1.15%, 80.90 ± 1.11%, respectively; $F_{1,80} = 58.77$, $P < 0.001$), and in 400 than

TABLE 1. Actual means (±1 SE) for variables presented as standardized means in Figs. 1, 4, and 6 for replicate experiments conducted in 2002 and 2003.

Atrazine	Embryos hatching (%)	Surviving until day 16 (%)	Day of hatching	Larvae in refuge (%)	Day of metamorphosis	Snout–vent length at metamorphosis (cm)
2002						
0 μg/L	98.39 ± 0.66	97.58 ± 0.94	6.66 ± 0.12	80.49 ± 3.18	65.67 ± 0.70	2.80 ± 0.02
4 μg/L	98.12 ± 1.21	97.58 ± 1.72	6.46 ± 0.17	77.77 ± 2.35	64.56 ± 0.57	2.79 ± 0.03
40 μg/L	96.77 ± 1.02	95.16 ± 1.21	6.58 ± 0.17	73.55 ± 3.27	64.14 ± 0.97	2.77 ± 0.02
400 μg/L	88.65 ± 2.94	88.38 ± 2.94	7.29 ± 0.20	74.98 ± 2.37	63.99 ± 0.64	2.70 ± 0.03
2003						
0 μg/L	93.13 ± 1.37	91.04 ± 1.71	8.77 ± 0.30	75.76 ± 2.73	63.58 ± 1.72	2.86 ± 0.06
4 μg/L	91.46 ± 1.22	88.75 ± 1.38	8.78 ± 0.25	74.39 ± 2.83	64.21 ± 2.00	2.89 ± 0.04
40 μg/L	89.38 ± 1.37	87.29 ± 1.62	8.77 ± 0.23	73.26 ± 3.21	58.30 ± 1.14	2.83 ± 0.05
400 μg/L	84.79 ± 1.97	81.25 ± 1.94	9.92 ± 0.40	70.02 ± 3.01	60.56 ± 2.02	2.82 ± 0.09

Note: There were no significant atrazine-by-year interactions for these variables.

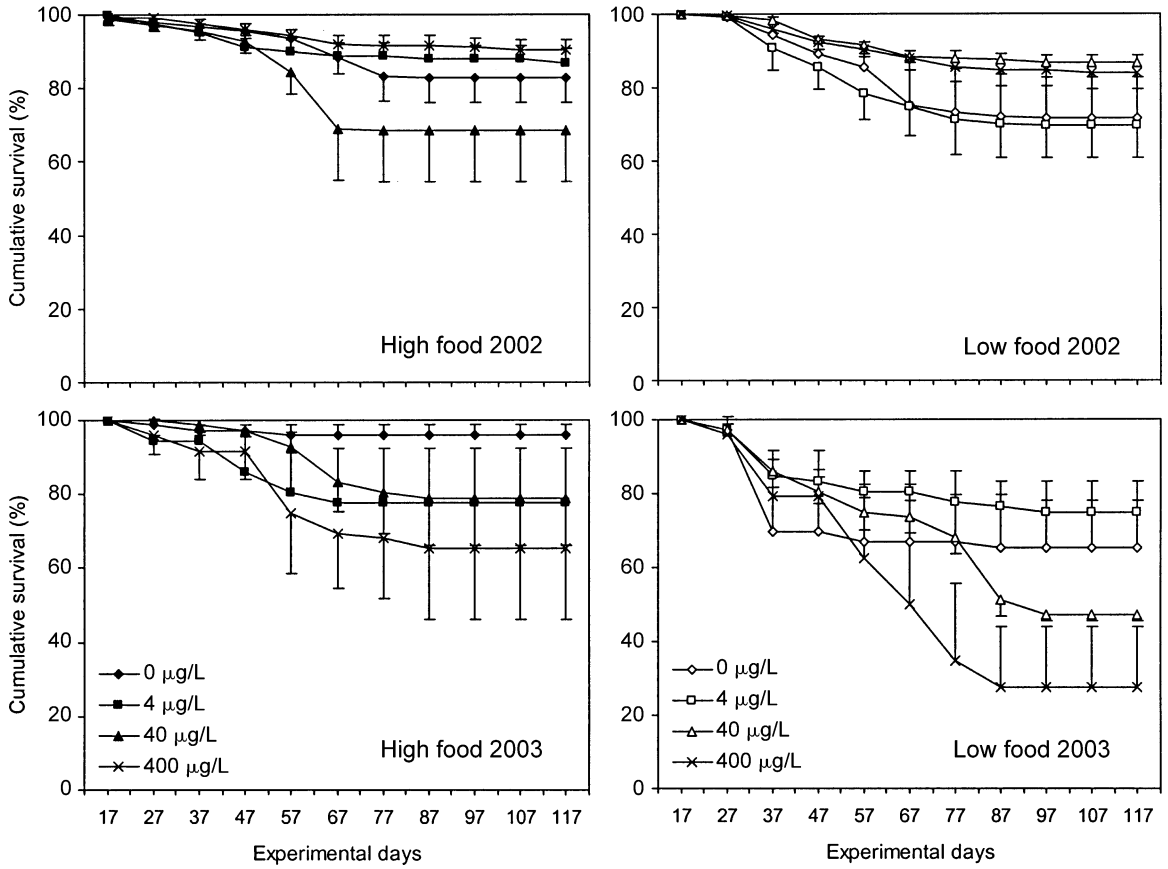

FIG. 2. Effects of atrazine (0, 4, 40, 400 μg/L) and food allotment (high and low) on cumulative percentage survival of *Ambystoma barbouri* after experimental day 16 for experiments conducted in 2002 and 2003. Plotted values represent means (±1 SE) of six replicate aquaria for 2002 and of three replicate aquaria (those that were not dried down) for 2003.

0 μg/L of atrazine ($F_{3,80} = 2.88$, $P = 0.041$; HSD, $P < 0.034$; other $P > 0.124$; Fig. 4 and Table 1). There were no significant interactions ($F < 0.81$, $P > 0.400$).

In 2002, we noticed that larvae exposed to higher concentrations of atrazine seemed to respond more often to our approach by vigorously swimming (J. R. Rohr, *personal observation*), but ''froze'' soon after. Because we imposed a one-minute wait after any apparent startle response, we feared that our measure of activity in 2002 was biased toward lower activity with higher atrazine concentrations. To obtain more accurate measurements of ambient activity, we only recorded activity in 2003 if the larvae did not appear to be disturbed upon our approach. This methodological change resulted in a significant atrazine-by-year interaction ($F_{3,80} = 7.08$, $P < 0.001$), but not a significant main effect of atrazine ($F_{3,80} = 0.62$, $P = 0.603$). In 2002, atrazine concentration was correlated negatively with larval activity ($R = 0.43$, $F_{1,46} = 10.49$, $P = 0.002$; Fig. 5), where in 2003, atrazine concentration was correlated positively with activity ($R = 0.382$, $F_{1,46} = 7.84$, $P = 0.007$). There were no other significant interactions ($F < 1.57$, $P > 0.215$), and food-limited larvae were significantly more active than larvae fed ad libitum (mean ± 1 SE: 9.57 ± 0.54%, 7.66 ± 0.44%, respectively; $F_{1,80} = 9.75$, $P = 0.002$).

Effects on metamorphosis

In 2003, the metamorphic parameters for *A. barbouri* in two aquaria were significant and influential outliers (determined using leverage, DFFITS, and DFBETAS tests; Neter et al. 1996), and were thus not included in the analyses. One aquarium containing 400 μg/L of atrazine was likely an outlier because it had only two late metamorphs. The reason for the other outlier is unknown.

For larvae that did not experience a reduction in water level, body condition at metamorphosis, or the relationship between mass and SVL, was unaffected by metamorphosis day ($F_{1,76} = 0.73$, $P = 0.397$), atrazine ($F_{3,76} = 1.099$, $P = 0.355$), food ($F_{1,76} = 2.05$, $P = 0.157$), or year ($F_{1,76} = 0.42$, $P = 0.521$), and there were no significant interactions ($F < 1.81$, $P > 0.157$). In 2003, body condition did not differ between larvae in control and dry-down treatments ($F_{1,29} = 0.11$, $P = 0.740$), and dry down did not interact statistically with food level or atrazine ($F < 0.96$, $P > 0.382$). Because none of the factors strongly affected body condition

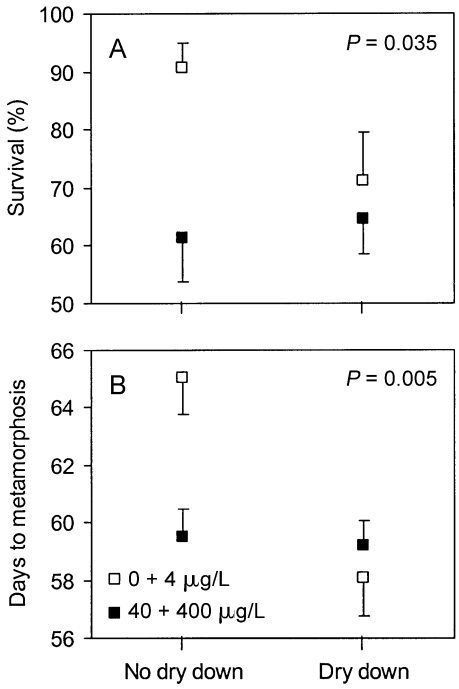

FIG. 3. Effects of atrazine (pooled 0 and 4 μg/L vs. pooled 40 and 400 μg/L) and hydroperiod (no dry down vs. a dry down) on (A) percentage of larval survival and (B) number of days to metamorphosis. Plotted values represent means (±1 SE) of six aquaria. Probability values are for the interaction between atrazine and hydroperiod.

and because metamorph mass and SVL were highly correlated ($r = 0.71$, $t_{1,91} = 92.40$, $P < 0.001$), we only present SVL analyses for brevity.

In aquaria that were not dried down, MANOVA showed that metamorphic parameters (day of, and SVL at, metamorphosis) were influenced by percentage larval survival (Wilks' $F_{2,51} = 7.23$, $P = 0.002$), mean day of larval mortality (Wilks' $F_{2,51} = 2.98$, $P = 0.060$), atrazine (Wilks' $F_{6,102} = 5.54$, $P < 0.001$), food abundance (Wilks' $F_{2,51} = 70.47$, $P < 0.001$), and year (Wilks' $F_{2,51} = 13.94$, $P < 0.001$). Food- and atrazine-by-year interactions were marginally nonsignificant (Wilks' $F_{2,51} = 3.11$, $P = 0.053$; Wilks' $F_{6,102} = 1.84$, $P = 0.099$, respectively). When controlling for timing of mortality ($F_{1,53} = 5.44$, $P = 0.023$), metamorphosis occurred later for food-limited than food-unlimited larvae (mean ± 1 SE: 65.28 ± 0.51 d, 62.02 ± 0.52 d, respectively; $F_{1,53} = 34.79$, $P < 0.001$) and for larvae exposed to 0 than 40 or 400 μg/L of atrazine ($F_{3,53} = 0.18$, $P < 0.001$; HSD, 0 vs. 40, $P = 0.002$, 0 vs. 400, $P = 0.05$; Fig. 6A and Table 1). Because atrazine and food abundance influenced survival and timing of metamorphosis and both these dependent variables were correlated positively with SVL at metamorphosis, we controlled for these variables when testing for the effects of atrazine and food limitation on metamorph SVL (Survival, $F_{1,52} = 13.07$, $P < 0.001$; Metamorphois day, $F_{1,52} = 8.33$, $P = 0.006$). When doing so, SVL was smaller for food-limited than food-unlimited larvae (mean ± 1 SE: 2.72 ± 0.02 cm, 2.86 ± 0.01 cm, respectively; $F_{1,52} = 63.75$, $P < 0.001$) and for larvae that were exposed to 400 than 0 μg/L of atrazine ($F_{3,52} = 3.48$, $P = 0.022$; HSD, $P = 0.004$; Fig. 6B and Table 1), indicating that both food limitation and 400 μg/L of atrazine reduced metamorph size independent of their effects on larval survival and timing of metamorphosis. Furthermore, this reduction in size occurred despite atrazine having no significant effect on larval feeding rates at any time during development in 2002 (mean ±1 SE: 87.86 ± 15.71, 92.05 ± 14.76, 72.35 ± 10.10, and 89.37 ± 6.56 mg consumed/larva for 0, 4, 40, and 400 μg/L, respectively; Atrazine, $F_{3,20} = 1.10$, $P = 0.371$; Atrazine × time: $F_{6,40} = 1.83$, $P = 0.117$).

In 2003, MANOVA revealed that metamorphic parameters (day of, and SVL at, metamorphosis) were influenced by percentage of larval survival (Wilks' $F_{2,36} = 10.80$, $P < 0.001$), dry down (Wilks' $F_{2,36} = 8.26$, $P = 0.001$), and food abundance (Wilks' $F_{2,36} = 39.80$, $P < 0.001$). In addition, atrazine interacted with dry down (Wilks' $F_{2,36} = 4.48$, $P = 0.018$), but all other interactions were nonsignificant (Wilks' $F_{2,36} < 1.89$, $P > 0.165$). When controlling for survival ($F_{1,37} = 9.90$, $P = 0.003$), larvae experiencing a dry down metamorphosed sooner than those not experiencing a dry down ($F_{1,37} = 8.39$, $P = 0.006$), but only if they were not exposed to 40 or 400 μg/L of atrazine (Atrazine × dry down, $F_{1,37} = 9.06$, $P = 0.005$; Fig. 3B). Dry down reduced metamorph size (mean ± 1 SE: 65.28 ± 0.51 cm vs. 62.02 ± 0.52 cm; $F_{1,36} = 6.81$, $P = 0.013$), even when controlling for survival ($F_{1,36} = 9.44$, $P = 0.004$) and timing of metamorphosis ($F_{1,36} = 4.81$, $P = 0.035$), indicating that drying conditions somehow decreased size above and beyond size reductions associated with reduced survival and accelerated metamorphosis. There was no

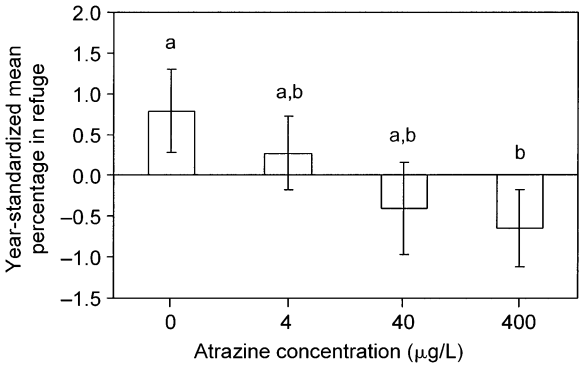

FIG. 4. Effects of atrazine (0, 4, 40, 400 μg/L) on percentage of larvae in refuge. There were no significant atrazine- or food-by-year interactions, so data were standardized using *z* scores (mean = 0, SD = 1) to account for differences between the two experimental years. Different letters above bars reflect significant differences among treatments according to Tukey's honestly significant difference multiple comparison tests. Bars represent means (± 1 SE) of 24 replicate aquaria (12 per year). See Table 1 for actual mean values.

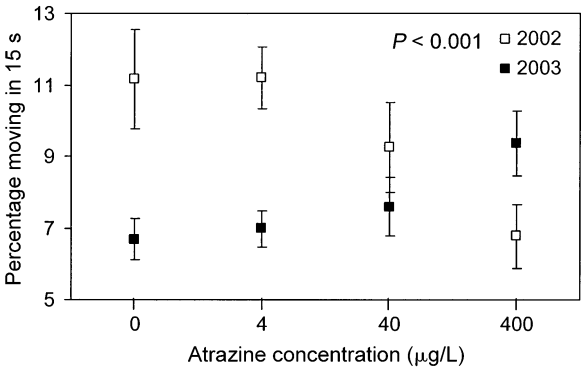

FIG. 5. Effects of atrazine (0, 4, 40, 400 μg/L) and year (2002 and 2003) on percentage of larvae moving during a 15-s interval. Plotted values represent means (±1 SE) of 12 replicate aquaria. The probability value is for the interaction between atrazine and experimental year. The difference in response to atrazine between years is probably due to a change in methodology. See *Results: Effects on larval activity and refuge use* for details.

atrazine-by-dry down interaction for SVL at metamorphosis ($F_{1,36} = 0.12$, $P = 0.731$).

Because atrazine seemed to elevate larval activity without concomitantly increasing feeding rates, elevated activity alone (presumably through metabolic costs) could have accounted for the reduced size at metamorphosis of atrazine-exposed animals. To test this hypothesis, we conducted an ANCOVA using SVL at metamorphosis as the dependent variable, atrazine, food level, and dry down, as the categorical predictors, mean day of metamorphosis, survival, and activity as our continuous predictors (all had $P < 0.05$), and an atrazine-by-activity interaction term to determine whether the relationship between activity and SVL depended on atrazine exposure. Only data from 2003 were used because we had more accurate estimates of ambient activity in this year. This analysis revealed that aquaria with more active larvae had smaller metamorphs (Activity: $F_{1,34} = 4.40$, $P = 0.043$). However, this relationship was independent of atrazine exposure (Atrazine × activity: $F_{1,34} = 0.50$, $P = 0.487$), indicating that atrazine-induced increases in activity could not solely account for the reduced size of *A. barbouri* exposed to atrazine.

Effects on water chemistry and quality

No atrazine was detected in control aquaria as determined by FID gas chromatography. Measured doses of atrazine were consistently similar to nominal doses (e.g., day 70, 4.95 ± 0.30 μg/L, 38.70 ± 2.14 μg/L, 386.04 ± 23.24 μg/L [means ± 1 SE]), and atrazine levels in exposure water measured immediately before and immediately after water changes demonstrated that atrazine levels in aquaria remained stable between weekly water changes (A. Elskus, *unpublished data*).

Results of MANOVA indicated that atrazine (Wilks' $F_{9,92} = 3.64$, $P < 0.001$), food abundance (Wilks' $F_{3,38} = 16.23$, $P < 0.001$), and time (Wilks' $F_{9,32} = 483.88$, $P < 0.001$) influenced water quality, and that water quality changed differently through time for different levels of atrazine (Atrazine × time: Wilks' $F_{27,94} = 1.91$, $P = 0.012$) and food (Food × time: Wilks' $F_{9,32} = 3.81$, $P = 0.002$). Water temperature was independent of atrazine and food abundance ($F < 0.05$, $P > 0.984$). DO was also lower in high-food 40 and 400 μg/L aquaria than in high-food 0 and 4 μg/L aquaria (mean ± 1 SE: 9.252 ± 0.015 mg/L, 9.338 ± 0.024 mg/L, respectively; $F_{3,40} = 3.43$, $P = 0.026$), probably because algae were visibly less abundant in aquaria with 40 or 400 μg/L of atrazine. The pH sharply decreased through the experiment (from 7.865 ± 0.007 to 7.116 ± 0.029 [mean ± 1 SE]; $F_{3,120} = 843.42$, $P < 0.001$) but declined more sharply for high-food and for low-atrazine concentration aquaria (Food × time: $F_{3,120} = 19.03$, $P < 0.001$; Atrazine × time: $F_{9,120} = 6.93$, $P < 0.001$).

These differences in water quality between treatments were small and the ranges of all water quality measurements (Temperature, 15.1°–17.0°C; DO, 8.7–

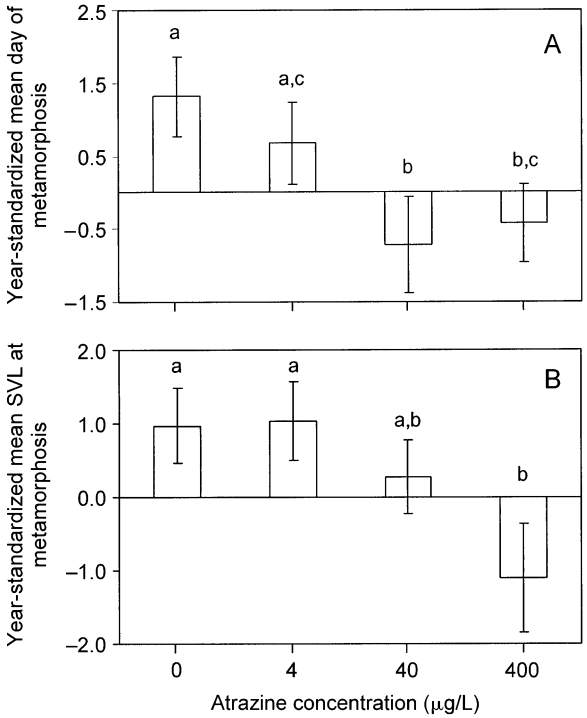

FIG. 6. Effects of atrazine (0, 4, 40, 400 μg/L) on (A) day of metamorphosis and (B) snout–vent length (SVL) at metamorphosis. There were no significant atrazine- or food-by-year interactions, so data were standardized using *z* scores (mean = 0, SD = 1) to account for differences between the two experimental years. Different lowercase letters above bars reflect significant differences among treatments according to Tukey's honestly significant difference multiple comparison tests. Bars represent means (±1 SE) of 18 replicate aquaria (12 in 2002 and six in 2003). See Table 1 for actual mean values.

9.7 mg/L; pH, 6.67–7.94) were within the acceptable guidelines for toxicity tests on amphibians outlined by the American Society for Testing and Materials (ASTM 1996), suggesting that the water quality differences may not have been biologically significant. To assess the effect of DO and pH (at day 56 when differences were greatest) on *A. barbouri* size at, and time to, metamorphosis, and percentage survival, in refuge, and active, we regressed DO and pH against these dependent variables while controlling for the effects of atrazine and food abundance. The results of this MANCOVA revealed that there was no significant relationship between these dependent variables and DO (Wilks' $F_{5,36} = 0.06$, $P = 0.997$) or pH (Wilks' $F_{5,36} = 1.09$, $P = 0.384$), indicating that the statistically significant water quality differences between treatments in 2002 were not responsible for treatment differences in growth, life history, or behavior.

Discussion

Our results demonstrate that atrazine, food abundance, and hydroperiod have the potential to influence *A. barbouri* population dynamics by altering their survival, life history, and behavior. More specifically, (1) atrazine had substantial effects on embryo survival and hatching, (2) atrazine, food limitation, and drying conditions reduced larval survival and altered metamorphic variables, (3) atrazine and food abundance affected larval behavior, and (4) atrazine had opposing effects on different life-history stages, emphasizing the importance of considering responses through ontogeny.

Embryos

Embryos exposed to 400 μg/L of atrazine were less likely to hatch than those exposed to 0 μg/L of atrazine, providing the first example of an ecologically relevant concentration of atrazine inducing significant amphibian embryo mortality. However, our reduced embryo survival is contrary to the results of Allran and Karasov (2001) who found that embryo hatching of various anuran species (*Rana pipiens*, *R. sylvatica*, and *Bufo americanus*) was unaffected by atrazine concentrations 500 times our highest dose. Species differences in amphibian tolerance to pesticides have been well documented within the order Anura (Berrill et al. 1998, Bridges and Semlitsch 2000), and thus differences in tolerance may be even more likely when comparing anurans to *A. barbouri*, a member of the order Caudata. In addition, animals with restricted ranges are thought to be narrowly adapted and more sensitive to contaminants (Fioramonti et al. 1997), which may explain the greater susceptibility of *A. barbouri* embryos to atrazine than the widespread anuran species tested by Allran and Karasov (2001).

The impact of atrazine-induced egg mortality on *A. barbouri* population dynamics probably depends on the density of larvae in particular years. A model examining population-level consequences of amphibian egg mortality suggests that species with strong density-dependent larval survival may have populations that are relatively insensitive to mortality at the egg stage (Vonesh and De la Cruz 2002). During most years in the field, the survival of *A. barbouri* larvae is strongly density dependent (Petranka and Sih 1986), suggesting that, in general, increased egg mortality may have only weak effects on population dynamics. However, atrazine-induced egg mortality could be devastating to adult recruitment in years when heavy spring floods wash away most larvae (up to 90%), leaving only a small percentage that are much less impacted by density effects (Petranka and Sih 1986).

Larvae

In 2002, larval survival of *A. barbouri* was independent of atrazine exposure, consistent with results from other studies where amphibian larvae were exposed to concentrations of atrazine as high as 20 mg/L (Diana et al. 2000, Allran and Karasov 2001, Rohr et al. 2003). However, in 2003, 400 μg/L of atrazine caused significant larval mortality, consistent with significant larval mortality of the African clawed frog, *Xenopus laevis*, exposed to an atrazine concentration of 320 μg/L (Sullivan and Spence 2003). The difference between the years in atrazine-related mortality suggests that the lethality of atrazine may be condition dependent for *A. barbouri*. Thus, the interactive effects of atrazine with abiotic factors not addressed by this study should be explored.

For larval treefrogs, *Hyla versicolor*, the threat of being consumed by a predator synergistically increased the lethality of the insecticide carbaryl (Relyea and Mills 2001), but for *A. barbouri*, neither desiccation nor starvation stress showed great potential to multiplicatively increase the lethality of atrazine. Drying conditions and atrazine interacted for larval survival, but the effect was in the opposite direction from our prediction. Drying conditions reduced larval survival for the pooled 0 μg/L and 4 μg/L atrazine group but did not affect survival for the pooled 40 μg/L and 400 μg/L group. Thus, the combined effects of stream drying and atrazine exposure may not pose a greater threat to *A. barbouri* larvae than either factor alone. In contrast, food limitation combined with atrazine did increase larval mortality relative to each lone factor, but this increase was generally additive rather than synergistic, as supported generally by a variety of other studies (e.g., Kluttgen and Ratte 1994, Bridges et al. 1997, Hopkins et al. 2002). Although feeding would be necessary to acquire energy to detoxify atrazine, it may also increase atrazine intake because atrazine has been shown to bioconcentrate (Allran and Karasov 2000, Nikkila et al. 2001). Larval cannibalism was observed occasionally in our study, and thus, if the biomagnification potential of atrazine is low, cannibalism may have prevented larvae from entering a state of negative energy balance where they may be more likely

to succumb to atrazine exposure. Because resources are often limited in natural systems, and may even be reduced in contaminated habitats, greater effort should be devoted to identifying which species are most susceptible to simultaneous resource limitation and contaminant exposure, and which contaminants are inclined to increase in lethality with decreasing resources.

In addition to their similar effects on survival, food limitation and atrazine had parallel effects on *A. barbouri* behavior. Both factors tended to reduce larval refuge use and increase larval activity. These behavioral changes for food-limited larvae have been demonstrated previously for *A. barbouri* under hunger stress (Rohr et al. 2003), and should increase encounter rates with food. The atrazine-enhanced tendency to swim and then ''freeze'' in response to certain visual or vibration stimuli that we observed in 2002 is consistent with increased burst swimming reactions in our earlier study on *A. barbouri* (Rohr et al. 2003) and in work on fish (Macek et al. 1976, Saglio and Trijasse 1998). However, the activity measurements in 2002 did not seem to reflect larval activity when undisturbed. In 2003, when our activity measurements more accurately reflected ambient levels, there was a positive correlation between atrazine concentration and activity. Although the atrazine-induced reduction in refuge use and increase in activity did not appear to strongly influence feeding rates, they may elevate predation risk by increasing conspicuousness and encounters with predators.

Metamorphs

Atrazine and food restriction reduced size at metamorphosis, but had opposing effects on larval duration, with atrazine decreasing, and food limitation increasing, the length of the larval period. Pesticides have often been shown to reduce amphibian growth (e.g., Boone et al. 2001, Rehage et al. 2002), but have only occasionally reduced metamorph size (Larson et al. 1998, Diana et al. 2000) or accelerated metamorphosis (Cheek et al. 1999, Boone et al. 2001). Earlier metamorphosis may provide a benefit to atrazine-exposed animals by reducing atrazine exposure and desiccation risk from stream drying, but their smaller size at metamorphosis could result in an overall detrimental effect of atrazine, because, for many amphibians, smaller metamorphs have lower terrestrial survival, lower lifetime reproduction, and compromised immune function (Berven and Gill 1983, Smith 1987, Berven 1990, Scott 1994, Carey et al. 1999). The smaller size at metamorphosis of atrazine-exposed and food-limited larvae could decrease the rate of recruitment to the breeding adult population, which would reduce population sizes (Semlitsch et al. 1988). Of greater concern is that exposure to atrazine may be more detrimental in food-limited populations or in years with limited resources, because the smallest metamorphs came from food-limited aquaria with high atrazine concentrations. This provides further support that natural stressors can enhance anthropogenic risks accelerating amphibian declines (e.g., Relyea and Mills 2001, Blaustein and Kiesecker 2002). Some populations of *A. barbouri* seem to lack sufficient immigration to adequately compensate for declines and have limited genetic variation on which selection can act (Storfer 1999), suggesting that these life-history shifts may have the potential to lead to local extinctions.

Unlike food limitation, which delayed metamorphosis, drying conditions accelerated metamorphosis, but only for the pooled 0 μg/L and 4 μg/L atrazine group. Because 40 μg/L and 400 μg/L of atrazine tended to speed up larval development (but slowed growth), many of these larvae may have been near their physiological limit for accelerating metamorphosis and incapable of further acceleration when experiencing drying conditions. However, these larvae did not seem to be at any greater risk of desiccation than larvae exposed to 0 μg/L or 4 μg/L of atrazine because, on average, they metamorphosed at similar times. It should be noted that our design for the dry-down experiment allowed for selection and physiological and growth divergence to occur prior to drying, and the effects of these changes on metamorphic parameters are not clearly understood. Interestingly, atrazine and drying reduced metamorph size independent of their effects on survival and timing of metamorphosis, indicating that the reduced competition and accelerated metamorphosis associated with these stressors did not compensate for their adverse effects on growth.

Our data, and those of others, allow us to consider possible mechanisms for the reduced size at metamorphosis of *A. barbouri* exposed to atrazine. Larvae exposed to 400 μg/L of atrazine had feeding rates comparable to control larvae, so a reduction in foraging rate could not explain their reduced growth rate. Increased energy expenditure from elevated activity, reduced feeding duration due to a shortened larval period, and changes in competition for food associated with mortality also could not fully account for the reduced size of atrazine-exposed animals. The cause of this size reduction is likely the disruptive effect of atrazine on neuroendocrine processes that are not directly related to activity and feeding. Larson et al. (1998) showed that a concentration of atrazine similar to our highest dose (250 μg/L) altered thyroxine levels of tiger salamanders, *Ambystoma tigrinum*, which reduced their size at metamorphosis. Larson et al. (1998) suggested that atrazine decreased the efficiency of energy assimilation into growth (possibly due to a diversion of energy for detoxification), consistent with the different growth rates but similar body conditions and feeding rates of our control and atrazine-exposed animals. However, atrazine did not accelerate tiger salamander metamorphosis (Larson et al. 1998) contrary to our results, the effect of another herbicide on amphibian

metamorphosis (Cheek et al. 1999), and the predicted effect of stressors on metamorphic timing (see next paragraph; Wilbur and Collins 1973). A delay in metamorphosis would increase the duration of contaminant exposure, which, under certain conditions, could increase mortality late in experiments (as we saw in 2003) truncating metamorphosis distributions. This may partly explain why timing of amphibian metamorphosis is highly variable across studies of the same contaminant. Clearly, more studies are needed to fully understand the effects of atrazine, and pesticides in general, on the neuroendocrine control of amphibian life histories.

Amphibian metamorphosis models may have value in predicting and interpreting the effects of toxicants on metamorphic parameters because contaminants may provoke a stress response analogous to competition. For example, the accelerated metamorphosis at a small size of *A. barbouri* exposed to atrazine is consistent with the Wilbur and Collins' (1973) prediction that amphibians found in a highly stressful environment, and at a size capable of metamorphosis, should transfer energy from growth to differentiation to escape the stress. Despite their potential value in improving our understanding of how life histories may be re-shaped by exposure to contaminants, these models have yet to be generally applied to studies of amphibian ecotoxicology (but see Larson et al. 1998).

Contaminants, ontogeny, and future directions

Categorizing the effects of a stressor into various life-history stages can be logistically and organizationally useful, but it is important to consider the effects across ontogeny because they may differ with developmental stage. For instance, susceptibility to atrazine differed between embryo and larval life-history stages, consistent with other amphibian studies demonstrating that the timing of pesticide exposure during ontogeny can influence its effects (e.g., Berrill et al. 1998, Boone et al. 2001, Rohr et al. 2003). Additionally, embryos exposed to 400 μg/L of atrazine tended to hatch later than control embryos, which could prolong time in streams and result in catastrophic mortality from stream drying or from aquatic predation (Petranka and Sih 1986, Sih et al. 1992). However, atrazine exposure decreased the duration of the larval period, which presumably would decrease time in streams and counteract the increased desiccation risk from delayed hatching. Whether accelerated metamorphosis was induced by chronic atrazine exposure or exposure at a particular developmental stage cannot be determined from our experimental protocol. But this distinction is critical in determining how atrazine exposure in early development might affect desiccation risk, especially in light of atrazine being applied to croplands in early spring as a pre-emergent herbicide (Solomon et al. 1996).

Multiple stressors can also have opposing effects, emphasizing the need to consider this complexity before estimating net effects in nature. For example, food limitation prolonged, while atrazine decreased, the duration of the larval period making the risk of desiccation under these concurrent stresses difficult to predict. Furthermore, quantifying the persistence of contaminant-induced effects is critical to understanding their impact on amphibian fitness because stressors are thought to play more important roles in amphibian population declines if their effects are felt later in ontogeny (Vonesh and De la Cruz 2002). For instance, while many studies have reported changes in motor activity associated with atrazine exposure (Macek et al. 1976, Podda et al. 1997, Saglio and Trijasse 1998), the persistence of these activity changes remain unexplored. If alterations in activity persist after atrazine exposure or are permanent, as are the effects of atrazine on the reproductive system of frogs (Hayes et al. 2002*b*), they could affect predation, feeding, growth, osmoregulation, and, ultimately, survival at crucial late developmental stages. Consequently, the post-exposure effects of atrazine on juveniles reared in various environmental conditions are under investigation.

The relationship between the results of our laboratory study and the effects of atrazine on actual amphibian population dynamics is unclear. However, our work suggests that the upper range of atrazine concentrations found in nature has potential to be detrimental to amphibians at various stages in development.

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