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AQUATIC HERBICIDE EXPOSURE INCREASES SALAMANDER DESICCATION RISK EIGHT MONTHS LATER IN A TERRESTRIAL ENVIRONMENT

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Abstract—Contaminants and climate change may be factors in amphibian declines. However, few studies have explored their joint impacts on postmetamorphic amphibians, a life stage of great importance to amphibian population dynamics. Here, we examine the effects of premetamorphic exposure (mean exposure of 64 d) to ecologically relevant concentrations of the globally common herbicide atrazine (0, 4, 40, 400 μg/L) on the behavior and water retention of lone and grouped postmetamorphic, streamside salamanders, *Ambystoma barbouri*. Salamanders exposed to ≥40 μg/L of atrazine exhibited greater activity, fewer water-conserving behaviors, and accelerated water loss four and eight months after exposure compared to controls. No recovery from atrazine exposure was detected and its effects were independent of the presence of conspecifics. These results are consistent with the hypothesis that adverse climatic conditions and contaminants can interact to harm post-metamorphic amphibians; however, they suggest that these two stressors need not be experienced simultaneously to do so. These results emphasize the importance of considering both latent and cumulative effects of temporally linked stressors in ecotoxicology.

Keywords—Amphibian declines Climate Ontogeny Pesticide Post-exposure effects

INTRODUCTION

Exposure to pesticides is one of many factors that may be contributing to the global decline of amphibians [1–3]. Amphibians are thought to be sensitive to pesticides [4] because their highly permeable integuments may provide little resistance to contaminant uptake. Widespread and abundant pesticide use also makes it likely that amphibians will encounter pesticides. Over 63,000 pesticides are registered in the United States alone [5] and global pesticide use is estimated at approximately 2.5 billion kg of active ingredients per year [6]. Amphibian declines in the western United States recently have been correlated with downwind pesticide use [7,8], and pesticide residues have been deposited atmospherically at the earth's poles [6], suggesting that contaminants might have the potential to contribute to declines in remote and so-called pristine habitats.

In addition to potentially making amphibians sensitive to contaminants, their permeable skin also makes many amphibian species susceptible to evaporative water loss [9]. In fact, many amphibian population declines have been linked to droughts (reviewed by Boone et al. [10] and Carey and Alexander [11]) and some have been associated with dryness attributed to global climate change [12,13]. Though both pesticides and drying conditions have the potential to stimulate declines on their own, their joint effects may be far worse. Pounds and Crump [14] postulated that dryness associated with global climate change may interact with contaminant exposure to accelerate amphibian declines. Indeed, most amphibian declines are presumed to be due to interactions among multiple stressors [3]. Nevertheless, few studies have examined the combined effects of contaminants and drying conditions (but see [15–17]), and none have considered their joint effects on postmetamorphic amphibians, a life-history stage that typically has greater impacts on amphibian population dynamics than the more commonly studied embryo and larval stages [18,19].

The vast majority of multiple stressor studies have quantified the effects of simultaneous stressor exposure, despite sequential exposure being more common in nature. This is of great concern for amphibians because embryo and larval environments commonly affect juvenile and adult performance (e.g., [20]), and thus stressors experienced early in life may interact with stressors experienced later (e.g., [21]). In fact, two recent reviews of amphibian multiple stressor studies [2,22] concluded that, to fully understand how amphibians are impacted by stress, we need more studies considering the cumulative effects of temporally linked stressors across life-history stages.

Here, we investigate the effects of exposure to the globally common herbicide atrazine [6] on the behavior and water retention of adult, streamside salamanders, *Ambystoma barbouri*, tested under drying conditions potentially associated with natural or anthropogenic climate variation. *Ambystoma barbouri* only were exposed to atrazine as embryos and larvae, but were tested approximately four and eight months later as terrestrial juveniles to determine how exposure early in ontogeny influences behavior and water retention in the critical later life-history stages. This allowed us to test for long-term, postexposure effects of atrazine that spanned life phases and major physiological (metamorphosis) and environmental changes. *Ambystoma barbouri* also were tested in the presence and absence of conspecifics to determine whether any effects of atrazine or dryness were dependent upon this context.

We hypothesized that *A. barbouri* previously exposed to atrazine would be hyperactive because atrazine has elevated motor activity in larval *A. barbouri* [15,23], in other amphibians [24,25], and in fish [26]. Because high activity can preclude water conserving behaviors and can increase exposed

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surface area [27], we expected atrazine-exposed *A. barbouri* to have a greater rate of water loss under dry conditions.

Pesticide background

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) is an inhibitor of photosynthesis that predominantly is used 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 [28,29]. Its region of heaviest use in the United States (Midwest) overlaps the range of *A. barbouri* and, in other amphibians, atrazine has been shown to be an endocrine disruptor [25,29].

Atrazine is relatively mobile [28], washing into water bodies during the embryo and larval development of many amphibians. It is also relatively persistent. Reported half-lives in ponds and mesocosms range from 95 to 350 d [30], indicating that long-term exposure is likely. Due to its stability and extensive use, atrazine is one of the most commonly found pesticides in the atmosphere and can be transported aerially up to a 1,000 km [6]. In Iowa, USA, mean wet deposition of atrazine was 7 μg/L, but values reached as high as 154 μg/L [6]. In surface waters, atrazine seldom exceeds 50 μg/L [28], but in water bodies near agricultural areas, atrazine levels have been reported at and above 500 μg/L [31,32].

MATERIALS AND METHODS

Ambystoma barbouri used in this study were metamorphs from a 2002 experiment (see [15] for detailed methodology) where 31 eggs (from a pool of 38 homogenously mixed clutches) were distributed into each of 48 aquaria containing 9.5 L of dechlorinated, constantly aerated tap water. These aquaria were maintained at 15°C on 12:12-h photoperiod and their water was changed weekly. After hatching, *A. barbouri* were reared on blackworms, *Lumbriculus variegatus*.

Ambystoma barbouri were exposed to either 0, 4, 40, or 400 μg/L of atrazine from embryo stage to metamorphosis (mean exposure of 64 d, see [15]), a potentially ecologically relevant exposure regime (see *Pesticide background* section). The concentrations were verified regularly throughout the exposure period [15]. After metamorphosis, *A. barbouri* were housed in moist terraria, fed vitamin- and mineral-enriched crickets weekly (ad libitum), and maintained at room temperature (20°C) under a 12:12-h photoperiod.

On October 14, 2002, a mean of 130 d (standard deviation [SD] = ±2.98; range 88–139) since *A. barbouri* were last exposed to atrazine treatments (i.e., since metamorphosis), metamorphs from each atrazine treatment were selected randomly and distributed either alone or in groups of three among 96 10-cm-diameter Petri dishes (see *Results* section for mean starting mass of salamanders in each treatment). These dishes were placed on two shelves (due to confined space) in an environmental chamber maintaining 26°C, the mean summer soil temperature in forests at a similar part of the country [33]. Each Petri dish was lined with filter paper and weighed (±0.001 g) before and immediately after receiving metamorphs. Every hour thereafter for the next 5 h, we recorded the mass of the metamorphs (by weighing each dish; mass of groups was divided by three), the number of salamanders in each dish that were moving and pressed against the dish side, and the number of animals huddled (greater than 50% of the side of their body in contact with a conspecific) for dishes containing three metamorphs. These behaviors were quantified because they can affect water economy by modifying exposed surface area [27]. We recorded mass loss every hour so we could determine whether the water loss curve appeared near an asymptote. All observations occurred between 0900 and 1500 h, and a second replicate trial was conducted one week later without retesting animals. Consequently, we employed a completely randomized 4 × 2 factorial design with 24 replicates (12 in each trial) of each atrazine by conspecific combination.

The protocol just described was used to retest the same pool of salamanders on January 29 and 31, 2003, a mean of 237 and 239 d since last atrazine exposure. However, all salamanders were tested alone and two metamorphs were removed from the analyses because they escaped from their dishes. Trials conducted 130 and 137 d after metamorphosis are referred to as the early ontogeny (EO) block and those conducted 237 and 239 d after metamorphosis are referred to as the late ontogeny (LO) block. Experiments lasted for only 5 h to prevent mortality so that the effects of atrazine on the reproductive system of *A. barbouri* could be quantified once they reach sexual maturity.

For most statistical analyses, the two ontogenetic blocks were analyzed separately because we did not keep track of individuals tested during each block and because groups of metamorphs were tested only in the EO block. Although these blocks were not entirely independent, in one analysis, we treated ontogenetic block as a categorical predictor to assess whether there was recovery from atrazine exposure. We employed a repeated-measures, regression-based, multivariate analysis of variance (using a general linear model) to test for overall interactions and main effects of atrazine (a continuous predictor) and conspecifics (a categorical predictor) on the percent of metamorphs active and against the dish side in hours 1 through 3 versus 4 through 5 (results did not substantially differ if we compared the means for hours 1–2 to 3–5). This was followed by repeated-measures, univariate analyses of variance to examine independently activity, use of dish side, huddling, and the percent of mass lost per hour. Where the main effect of atrazine was significant ($p < 0.05$), we compared all atrazine concentrations to the control using a Dunnett's test (DT). In all analyses of variance, proportion data were transformed angularly, the Petri dish was treated as the experimental unit, and we controlled for salamander initial mass (covariate) and the shelf on which salamanders were tested (spatial block). Trial was excluded from subsequent analyses because there were no significant differences between the two trials conducted within each ontogenetic block.

Partial correlation analyses, partialing out the variation attributed to the conspecific treatment, was used to determine whether there was a relationship between activity and water loss and use of the dish side and water loss. Correlation analysis also was used to test for a relationship between huddling and water loss.

RESULTS

No salamanders died during these Petri dish trials. The effect of atrazine exposure did not differ between the EO and LO blocks for behavior (Atrazine × ontogeny: Wilks' $F_{2, 279} = 0.49$, $p = 0.616$) or mass loss (Atrazine × ontogeny: $F_{1, 280} = 0.01$, $p = 0.907$), indicating no detectable recovery from atrazine. Thus, the results in the following paragraph refer to both blocks.

Multivariate analysis of variance revealed that the percent of salamanders moving and pressed against the dish side were

Table 1. Results of multivariate analyses of variance (MANOVA) and analyses of variance (ANOVAs) testing the effects of atrazine exposure (0, 4, 40, 400 μg/L), conspecifics (one or three/dish), and time (mean of h 1–3 vs 4–5) on salamander behavior (activity and use of dish side) early and later in ontogeny (mean of 133.5 and 238 d postmetamorphosis, respectively) when controlling for salamander initial mass and the shelf on which salamanders were tested (top or bottom)

	Early ontogeny block						Late ontogeny block					
			ANOVAs[a]						ANOVAs			
	MANOVA[a]		Activity		Use of dish side		MANOVA		Activity		Use of dish side	
Effects	Wilks' F	p	F	p	F	p	Wilks' F	p	F	p	F	p
Initial mass (covariate)	1.89	0.153	1.91	0.169	0.66	0.419	4.72	0.010	0.13	0.714	8.45	0.004
Shelf (block)	2.68	0.071	3.34	0.069	0.50	0.479	4.17	0.017	7.94	0.005	2.17	0.143
Atrazine	5.15	0.007	8.36	0.004	5.44	0.021	4.34	0.014	7.53	0.007	2.92	0.089
Conspecifics	21.81	0.000	24.47	<0.001	5.78	0.017	—	—	—	—	—	—
Atrazine × conspecifics	1.72	0.181	1.39	0.240	3.10	0.080	—	—	—	—	—	—
Time	5.24	0.006	6.16	0.014	4.69	0.032	12.18	<0.001	23.69	0.000	4.92	0.028
Time × shelf	1.81	0.166	3.64	0.058	0.00	0.988	1.61	0.203	0.55	0.458	2.56	0.112
Time × initial mass	1.09	0.338	0.77	0.382	1.49	0.224	3.96	0.021	7.95	0.005	0.89	0.347
Time × atrazine	2.59	0.078	3.67	0.057	1.39	0.240	1.83	0.163	3.22	0.074	2.05	0.154
Time × conspecifics	6.33	0.002	2.58	0.110	10.47	0.001	—	—	—	—	—	—
3-way interaction	1.71	0.184	0.15	0.695	3.32	0.070	—	—	—	—	—	—

[a] The degrees of freedom for each effect are 2,185 for the MANOVAs and 1,186 for the ANOVAs. The effect of conspecifics was tested only early in ontogeny. See text for the influence of these same effects on salamander huddling.

affected by atrazine, conspecifics, and time (Table 1). As *A. barbouri* dehydrated through the experiment, they increased their huddling ($F_{1, 92} = 5.76$, $p = 0.018$), inactivity, and use of the dish side (Table 1, Fig. 1); in general, each behavior was effective at reducing water loss (Pearson correlations LO block: $r = -0.291$, $p = 0.004$, $n = 96$; $r = -0.370$, $p < 0.001$, $n = 192$; $r = -0.236$, $p = 0.001$, $n = 192$, respectively; EO block: $r = -0.406$, $p < 0.001$, $n = 190$; $r = -0.113$, $p = 0.123$, $n = 190$, respectively). Previous exposure to increasing concentrations of atrazine was associated with reductions in huddling (Atrazine: $F_{1, 92} = 4.19$, $p = 0.044$; Atrazine × time: $F_{1, 92} = 0.87$, $p = 0.354$), inactivity, and use of the dish side; these effects of atrazine generally were similar in hours 1 through 3 relative to 4 through 5 (Table 1, Fig. 1). In the EO block, only salamanders previously exposed to 400 μg/L of atrazine exhibited significantly less huddling, inactivity, and use of the dish side than those that had not been exposed to atrazine (DT: $p < 0.042$, other $p > 0.110$). In the LO block, metamorphs exposed to 400 (DT: $p = 0.001$) or 40 μg/L of atrazine (DT: $p = 0.008$; 0 vs 4 $p = 0.113$) were more active than controls.

Percent mass loss was greater for *A. barbouri* on the top than bottom shelf (presumably due to a temperature gradient, EO block only) and for salamanders with smaller initial masses (Table 2). We controlled for initial mass because there was a trend for atrazine concentration to be related inversely to initial mass in both EO (mass [g] ± standard error [SE], 0: 1.053 ± 0.037, 4: 1.031 ± 0.031, 40: 0.991 ± 0.038, 400: 0.989 ± 0.035; $F_{1, 190} = 1.01$, $p = 0.316$) and LO blocks (mass [g] ± SE, 0: 1.234 ± 0.063, 4: 1.116 ± 0.040, 40: 1.169 ± 0.037, 400: 1.085 ± 0.042; $F_{1, 188} = 2.79$, $p = 0.096$). Controlling for these variables revealed that rate of mass loss was greater for metamorphs tested alone than in groups and was affected positively by atrazine concentration (Table 2, Fig. 2A and B). *Ambystoma barbouri* that never were exposed to atrazine lost water mass at a slower rate than those previously exposed to either 400 (DT; EO and LO: $p < 0.001$) or 40 μg/L of atrazine (DT; EO: $p = 0.002$, LO: $p = 0.018$), and nearly at a slower rate than those exposed to 4 μg/L of atrazine (DT; EO: $p = 0.114$, LO: $p = 0.087$; Fig. 2A and B). The effect of atrazine

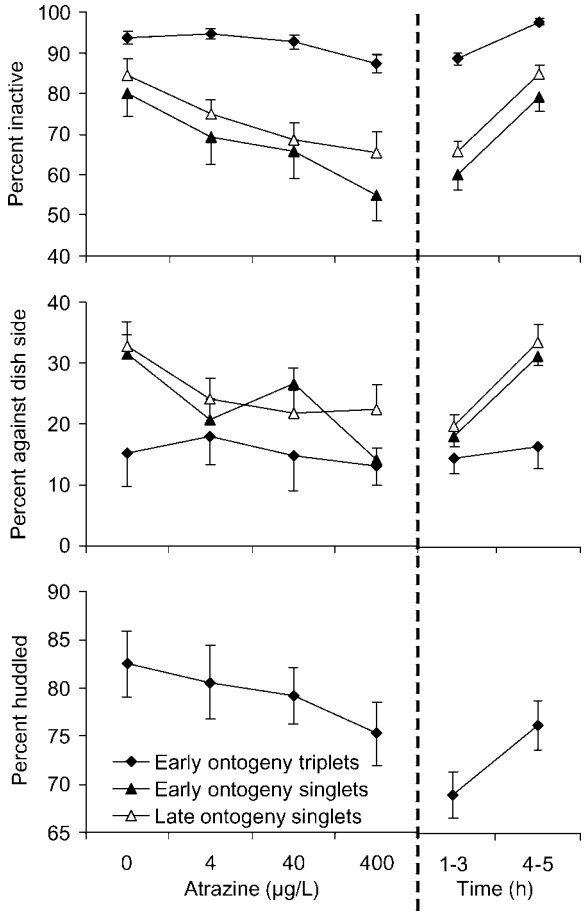

Fig. 1. Effects of previous atrazine exposure (0, 4, 40, 400 μg/L) and time (mean of hours 1–3 vs 4–5) on the percent of *Ambystoma barbouri* moving against the dish side, and huddling (>50% of body touching a conspecific) when tested early in ontogeny (mean 133.5 d since atrazine exposure and metamorphosis) or later in ontogeny (mean 238 d since atrazine exposure and metamorphosis). Early in ontogeny, *A. barbouri* were tested as triplets (three conspecifics per dish) and as singlets (alone in a dish), but later in ontogeny, they were only tested as singlets. In general, the effect of atrazine was independent of time. Symbols represent means (± standard error). See Table 1 and text for associated statistics.

Table 2. Results of analyses of variance testing the effects of atrazine exposure (0, 4, 40, 400 μg/L), conspecifics (one or three/dish),[a] and time (0–5 h) on salamander mass loss early and later in ontogeny (mean of 133.5 and 238 d postmetamorphosis, respectively) when controlling for salamander initial mass and the shelf on which salamanders were tested (top or bottom)

		Early ontogeny block		Late ontogeny block	
Effects	*df*	*F*	*p*	*F*	*p*
Initial mass (covariate)	1, 186	171.23	<0.001	38.89	<0.001
Shelf (block)	1, 186	12.98	<0.001	1.06	0.304
Atrazine	1, 186	6.82	0.010	7.10	0.008
Conspecifics	1, 186	701.99	<0.001	—	—
Atrazine × conspecifics	1, 186	0.27	0.601	—	—
Time	5, 930	1,265.95	<0.001	538.30	<0.001
Time × initial mass	5, 930	141.62	<0.001	28.04	<0.001
Time × shelf	5, 930	14.61	<0.001	4.92	<0.001
Time × atrazine	5, 930	6.75	<0.001	4.15	<0.001
Time × conspecifics	5, 930	621.73	<0.001	—	—
3-way interaction	5, 930	0.27	0.927	—	—

[a] The effect of conspecifics was tested only early in ontogeny.

on water loss was independent of conspecifics (Table 2). The slope of the mass loss curves did not decrease substantially during trials (Fig. 2A and B), suggesting that they were not close to the asymptotes and that, on average, the salamanders were not near critical water loss.

DISCUSSION

The results of this study demonstrate that embryonic and larval exposure to atrazine, one of the most common, persistent, and mobile herbicides in the world [6], can alter the behavior and increase the dehydration rate of postmetamorphic *A. barbouri* tested under conditions potentially associated with natural or anthropogenic climate variation. The mechanism for these effects is unknown, but atrazine has been shown to disrupt neuroendocrine processes in amphibians [24,29,34] and, thus, it may have altered neuroendocrine development associated with the expression of water-conserving behaviors. During the exposure period, increasing concentrations of atrazine increased embryo (but not larval) mortality [15], which could have selected for animals that were less likely to exhibit water-conserving behaviors after metamorphosis. Regardless of the mechanism, atrazine clearly affected behaviors tied to water retention, and these effects were independent of conspecifics, despite conspecific huddling substantially reducing salamander dehydration rates.

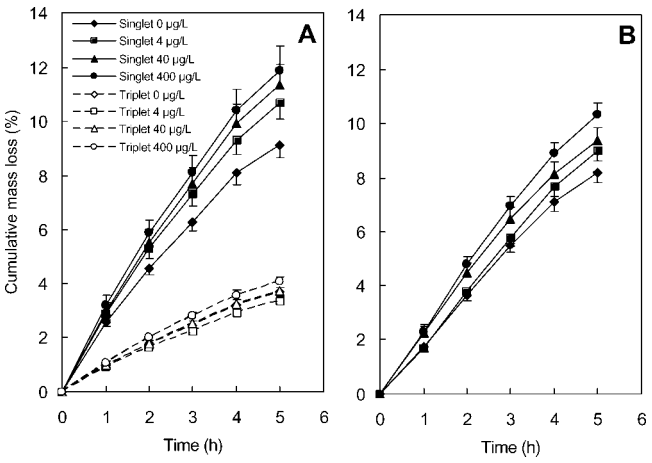

Fig. 2. Effects of previous atrazine exposure (0, 4, 40, 400 μg/L) and time (0–5 h) on the cumulative percent of *Ambystoma barbouri* mass loss when tested (**A**) early and (**B**) later in ontogeny (mean of 133.5 and 238 d since last atrazine exposure, respectively). Early in ontogeny *A. barbouri* were tested as triplets (three conspecifics per dish) and as singlets (alone in a dish). Symbols represent means (± standard error). See Table 2 for associated statistics.

Extrapolating from elevated rates of short-term water loss under laboratory conditions to effects of atrazine on wild populations clearly is a stretch; however, this study does raise some important toxicological and conservation concerns and lessons. The highly permeable integuments of most amphibians make them vulnerable to moderate increases in desiccation risk [9]. In fact, global climate change and widespread loss of forests and dispersal corridors already may be placing juvenile and adult amphibians at risk of dehydration during interhabitat movements [33]. Concerns for this taxon certainly must be heightened given that the globally common atrazine reduced amphibian water retention at 40 μg/L, a concentration found regularly in agricultural landscapes [31,32,35], and nearly reduced water retention at 4 μg/L, only 1 μg/L greater than what is allowed in U.S. drinking water [36].

Perhaps most notable is that the effects of atrazine on behavior and water retention occurred nearly eight months after atrazine exposure without any detectable recovery. Eight months should be long enough to detoxify and excrete atrazine, suggesting that the effects may be permanent. Exposure to high concentrations of contaminants probably is more likely for amphibians before metamorphosis because most amphibian embryos and larvae are strictly aquatic and cannot readily escape water bodies where many contaminants accumulate and concentrate. Thus, effects of aquatic atrazine exposure that carry over into the terrestrial stage are a significant finding. It suggests that neither simultaneous exposure to contaminants and dryness nor postmetamorphic exposure to contaminants may be necessary for these two factors to work in concert to harm postmetamorphic amphibians, a stage that often disproportionately affects amphibian population dynamics [18,19]. Clearly, there is a critical need for further amphibian experiments investigating interstage interactions of contaminants and dryness.

Effects of early life-stage exposure to atrazine on the desiccation risk of subsequent life stages highlights the importance of considering the cumulative effects of temporally linked stressors in ecotoxicology. If multiple stressors are in-

corporated into experiments at all [3], animals often are exposed to stressors concurrently, which may not be as common in nature as stressors being experienced sequentially. Although quantifying long-term or carry-over effects of stress experienced early in ontogeny is customary in psychology and common in ecology [21], it is, at best, occasional in conservation biology and toxicology [37]. This is disconcerting because long-term, sublethal effects of stressors may have greater consequences on populations than transient effects and, thus, may have superior potential for explaining population-level changes. Not surprisingly, some of the most catastrophic effects of contaminants on wildlife and human populations have been associated with long-term, postexposure effects, such as the enduring effects of DDT and the delayed toxicity of a variety of metals [37–39].

In addition, focusing on single life stages or short-term effects may give erroneous impressions that certain anthropogenic disturbances are innocuous. For example, Rohr et al. [15] found no evidence that atrazine affected desiccation risk for *A. barbouri* larvae, but this study suggests that atrazine can increase desiccation risk for *A. barbouri* after metamorphosis. Although it can be challenging to quantify environmental stress across temporal scales [40], characterizing the cumulative effects of temporally linked stressors and the lasting effects of stress experienced during formative developmental stages may be invaluable for understanding our present biodiversity crisis and for appropriately targeting conservation efforts.

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