

EFFECTS OF ATRAZINE AND IRIDOVIRUS INFECTION ON SURVIVAL AND LIFE-HISTORY TRAITS OF THE LONG-TOED SALAMANDER
(*AMBYSTOMA MACRODACTYLUM*)

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(Received 15 April 2005; Accepted 1 July 2005)

Abstract—Environmental contaminants and emerging infectious diseases are implicated as factors contributing to global amphibian declines. However, few studies have tested the interaction of these factors. We exposed six-week-old, larval long-toed salamanders (*Ambystoma macrodactylum*) to *Ambystoma tigrinum* virus (ATV; 0 or $10^{3.5}$ plaque-forming units/ml) and sublethal concentrations of atrazine (0, 1.84, 18.4, and 184 $\mu\text{g/L}$) in a 4×2 factorial design for 30 d. We tested the effects of atrazine and virus on mass and snout-vent length (SVL) at metamorphosis and larval period as well as on rates of mortality and viral infectivity. We confirmed ATV transmission to *A. macrodactylum* via polymerase chain reaction, but infection rates were lower than expected, consistent with the theory predicting lower pathogen transmission to nonnative hosts. Larvae exposed to both atrazine and ATV had lower levels of mortality and ATV infectivity compared to larvae exposed to virus alone, suggesting atrazine may compromise virus efficacy. The highest atrazine level (184 $\mu\text{g/L}$) accelerated metamorphosis and reduced mass and SVL at metamorphosis significantly relative to controls. Exposure to ATV also significantly reduced SVL at metamorphosis. The present study suggests moderate concentrations of atrazine may ameliorate effects of ATV on long-toed salamanders, whereas higher concentrations initiate metamorphosis at a smaller size, with potential negative consequences to fitness.

Keywords—Iridovirus Atrazine Contaminant Amphibian decline *Ambystoma macrodactylum*

INTRODUCTION

Amphibian populations are declining worldwide, with 48% of rapidly declining species being threatened by processes other than habitat destruction [1]. Emerging infectious diseases and environmental pollution are two of the leading hypotheses for these enigmatic declines [1,2]. Two emerging pathogens implicated in global amphibian epizootics are iridoviruses in the genus *Ranavirus* and a chytrid fungus (*Batrachochytrium dendrobatidis*) [1–3]. Although it is widely agreed that amphibian declines are not caused by a single factor [1,2], only recently have researchers begun to examine contaminant contribution to disease emergence in amphibians.

Environmental factors, such as agricultural contaminants, may play a role in disease emergence by suppressing the immune system [3,4]. For example, environmentally relevant mixtures of agricultural contaminants have been shown to increase susceptibility to infections in anurans by altering lymphocyte proliferation, spleen cellularity, and phagocytic activity of spleenocytes [4]. In addition, empirical studies have shown that pesticides can increase an amphibian's susceptibility to parasitic or bacterial infections. Trematode loads were higher, and limb deformities occurred more frequently, in wood frogs (*Rana sylvatica*) exposed to atrazine and malathion compared to those exposed to contaminant-free water [5]. Twice the number of adult lungworms (*Rhabdias ranae*) was found in the lungs of leopard frogs (*Rana pipiens*) exposed to a mixture of common pesticides compared to those exposed to lungworm alone [6]. When exposed to sublethal levels of malathion, Woodhouse's toads (*Bufo woodhousii*) were more susceptible to the bacterium *Aeromonas hydrophila* (the cause of

red-leg disease) compared to control animals [7]. These studies suggest that contaminants could have suppressive effects on the amphibian immune system, thereby increasing susceptibility to parasites and pathogens.

Ambystoma tigrinum virus (ATV) is an emerging iridovirus responsible for epizootics in tiger salamanders (*A. tigrinum*) throughout western North America. The virus was first isolated from San Rafael Valley, Arizona, USA, and ranges in distribution along the western cordillera into Saskatchewan and Manitoba, Canada [8–10]. The virus appears to have coevolved with its tiger salamander host [11], but it probably has been introduced to parts of the western United States through the use of barred tiger salamanders (*A. t. mavortium*) as fishing bait [9].

Ambystoma tigrinum virus is a monophyletic member of the genus *Ranavirus* [9]; ranaviruses are globally distributed pathogens of fish, amphibians, and reptiles and are associated with amphibian declines in the United Kingdom [12,13]. Phylogenetic data suggest that ATV may have originated from a host switch from game fish, such as largemouth bass, to tiger salamanders [9]. Therefore, a major concern is whether ATV will switch hosts again, infecting other amphibian species sympatric with *A. tigrinum* or species in areas where bait tiger salamanders have been introduced. In the Pacific Northwest, the long-toed salamander (*Ambystoma macrodactylum*) is sympatric with tiger salamanders at many sites. To our knowledge, ATV has not yet been confirmed in this region, but an iridovirus has been isolated from bullfrogs (*Rana catesbeiana*) in Washington (J. Evermann, Washington State University, Pullman, WA, USA, personal communication). Bullfrogs are both nonnative and invasive in Washington, and they have been expanding their geographic range. If ATV or another

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iridovirus is introduced by tiger salamanders or bullfrogs, it could potentially spread to *A. macrodactylum*.

Agricultural contaminants are a concern with emerging diseases, because they can facilitate disease emergence in new areas once the virus is spread or increase the severity of epizootics where the virus currently exists. Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-*S*-triazine) is one of the most widely used herbicides in the United States, with the greatest quantity being administered to corn crops during the spring months [14]. This period coincides with critical amphibian larval developmental stages [15]. Concentrations of atrazine in rivers can reach (but rarely exceed) 20 $\mu\text{g/L}$ between April and August [14]; however, concentrations of 200 $\mu\text{g/L}$ and greater occasionally have been detected [16]. Populations of salamanders in ponds and small streams adjacent to agricultural land are more likely to experience high concentrations from agricultural runoff [14], leaving ambystomatid salamanders particularly vulnerable, because they often breed in artificial wetlands, such as irrigation canals, farm ponds, and cattle tanks [17].

Atrazine has an acute toxicity of greater than 10,000 $\mu\text{g/L}$ in fishes, has a short half-life (3–90 d in water, depending on water salinity), and does not bioaccumulate significantly [14]. However, it is an endocrine disruptor, which can cause feminization of male leopard frogs at concentrations as low as 0.1 $\mu\text{g/L}$ [18]. Atrazine also has been shown to suppress prolactin and thyroid hormones [19], which are important hormones for immunomodulation. In amphibians, thyroid hormones, aided by corticosterone, also regulate amphibian metamorphosis [20–22]. Therefore, atrazine could play a role in disease emergence by suppressing amphibian immune systems or affecting normal growth rates.

The present study had three main objectives for better understanding the effects of ATV, atrazine, and possible interactions between the two. First, we tested whether *A. macrodactylum* is susceptible to ATV. If susceptible, we predicted that mortality from ATV should be reduced relative to *A. tigrinum*. This prediction is based on the theory that pathogens are locally adapted to their hosts because of shorter generation times and higher mutation rates [23]. Therefore, pathogen transmission should be attenuated in novel host species [23]. Second, we tested whether atrazine would alter *A. macrodactylum* life-history traits, including snout-vent length (SVL) and mass at metamorphosis and larval period. Third, we tested whether atrazine and ATV interact synergistically to affect mortality, viral infectivity, or life-history traits.

MATERIALS AND METHODS

Animal care

Approximately 200 eggs of *A. macrodactylum* were collected randomly at a high-altitude pond near St. Paul peak in the Cabinet Mountain Wilderness Area (Lincoln County, MT, USA; elevation, 1,436 m). Larvae were raised individually in round, polyethylene containers (12.7 \times 7.62 cm) containing 500 ml of artesian spring water aerated for a minimum of 24 h before water changes and treated with Reptisafe® (Zoo Med Laboratories, San Luis Obispo, CA, USA). Water was changed weekly before atrazine and ATV introduction. Larvae were fed 0.015 g of brine shrimp three times a week for six weeks. The larvae were then fed blackworms twice a week ad libitum beginning at six weeks of age (i.e., a few days before treatment exposure) and throughout the duration of the experiment. Larvae were reared on a 15:9-h light:dark cycle to mimic natural

conditions, and room temperature was maintained at $20 \pm 1^\circ\text{C}$ through the duration of the experiment.

Atrazine solutions

We dissolved 1.1561 g of 86.5% pure commercially available atrazine 90DF (Syngenta Crop Protection, Greensboro, NC, USA) in 1 L of artesian spring water. The solution was mixed thoroughly until all granules dissolved. One milliliter of this solution was added to 999 ml of artesian spring water to make a stock solution of 1 mg/L of atrazine. The stock was then diluted into four environmentally relevant treatment concentrations: 0, 2, 20, and 200 $\mu\text{g/L}$ [14,16]. The actual concentration of stock atrazine was 0.92 mg/L, as confirmed by liquid chromatography–mass spectrometry (University of Idaho Analytical Science Laboratory, Moscow, ID, USA). Thus, actual treatment concentrations of atrazine were 0, 1.84, 18.4, and 184 $\mu\text{g/L}$, respectively. A new stock solution was made up every 3 d immediately before each water change.

Experimental design

We used a complete 4×2 factorial design, with individually housed larvae assigned to one of four concentrations of atrazine (0, 1.84, 18.4, or 184 $\mu\text{g/L}$) and either none or $10^{3.5}$ plaque-forming units (PFU)/ml of ATV, which is the approximate dose necessary to result in 50% mortality (LD50) of tiger salamander larvae as determined by multiple experiments [11] (D. Schock, Arizona State University, Tempe, AZ, USA, unpublished data; A. Storfer, unpublished data). Larvae were randomly assigned to eight treatment groups (20 larvae/treatment, for a total of 160 individuals). Water was changed every 3 d to prevent degradation of atrazine, which has a minimum half-life of 3 d in water [14]. Mass (to the nearest 0.05 g) and SVL measurements (to the nearest 0.1 mm) were taken one week before treatment exposure and at metamorphosis (if larvae metamorphosed by 30 d). Larvae in ATV treatment groups were exposed to ATV (mixed with atrazine-treated water) during the first and second water changes only, for a total exposure period of 6 d, which was adequate for development of ATV infection based on previous experiments [11,24]. Animals were euthanized after 30 d to limit the possibility of sublethally infected larvae clearing virus. *Ambystoma tigrinum* larvae have been observed to clear viral infection after approximately 30 d [11] (A. Storfer, unpublished data). Tail clips were preserved in 95% ethanol and stored at -20°C , and whole bodies were frozen at -80°C . Tissue from tail clips was used for virus verification using polymerase chain reaction (PCR) primers to amplify the major capsid protein of ATV [25]. This protocol is sensitive enough to detect subclinical infection in animals, even from tail-clip tissues [11] (A. Storfer, unpublished data). The DNA was extracted using Gene Releaser® (BioVentures, Murfreesboro, TN, USA), and PCR conditions were as described by Jancovich et al. [9]. Presence of ATV was confirmed via visualization of the approximately 500-bp PCR product on agarose gels, and positive and negative controls were run in each PCR reaction.

Statistical analyses

We conducted all analyses using Systat 11.0© 2004 (Systat Software, Point Richmond, CA, USA). We tested the effects of atrazine, ATV, and their interaction on the following correlated response variables: Mass and SVL at metamorphosis and larval period using multivariate analysis of covariance. The SVL of salamanders taken one week before treatment

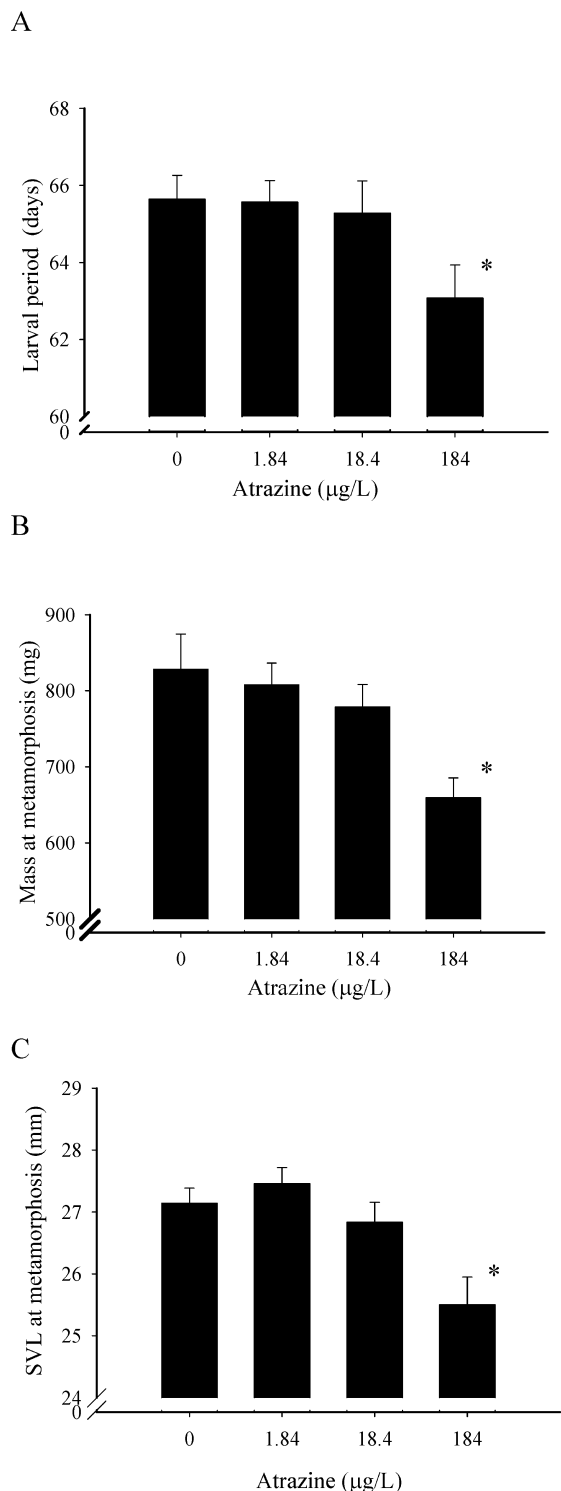


Fig. 1. (A) Effect of atrazine on larval period ($F = 2.76$; $df = 3, 98$; $p = 0.046$). Larval period is significantly decreased at 184 µg/L compared to all other treatments (Fisher's least-significant-difference test: 184–0 µg/L, $p = 0.014$; 184–1.84 µg/L, $p = 0.02$; and 184–18.4 µg/L, $p = 0.034$). (B) Effect of atrazine on mass at metamorphosis ($F = 5.521$; $df = 3, 91$; $p = 0.002$). Mass is significantly decreased at 184 µg/L compared to all other treatments (Fisher's least-significant-difference test: 184–0 µg/L, $p = 0.004$; 184–1.84 µg/L, $p < 0.001$; and 184–18.4 µg/L, $p = 0.003$). (C) Effect of atrazine on snout-vent length (SVL) at metamorphosis ($F = 7.341$; $df = 3, 96$; $p < 0.001$). Snout-vent length is significantly decreased at 184 µg/L compared to all other treatments (Fisher's least-significant-difference test: 184–0 µg/L, $p = 0.001$; 184–1.84 µg/L, $p < 0.001$; and 184–18.4 µg/L, $p = 0.003$). An asterisk indicates a significant result. Bar graphs indicate the mean + one standard error.

exposure served as a covariate to control for size variation among larvae. Analyses of covariance further tested for specific effects of treatments on each response variable, with multiple comparisons conducted using Fisher's least-significant-difference tests. We used log-linear analysis to test the effects of atrazine, ATV, and their interaction on the binomial variables of mortality and infectivity. Chi-square tests compared actual mortality in the ATV-only treatment against the 50% mortality rate expected under the null hypothesis. Chi-square tests also compared mortality in the ATV and ATV × atrazine treatments to the 50% mortality rate expected under the null hypothesis (use of ~LD50 dosage) and mortality and infectivity in the ATV × atrazine treatments to the actual mortality and infectivity rate in the ATV only treatment.

RESULTS

Transmission to *A. macrodactylum*

We observed disease symptoms and confirmed waterborne transmission of ATV to *A. macrodactylum* via PCR in 25% of animals exposed to virus alone. Symptoms of infection included lethargy, hemorrhaging in the extremities, sloughing of the skin, edema, and occasionally, a thick mucus appearing to originate from the cloaca. These symptoms are consistent with previous observations of ATV infection in tiger salamanders [10,24]. Individuals surviving to 30 d were negative for ATV infection. No cross-contamination of ATV occurred, because no individual within groups unexposed to ATV showed symptoms of ATV or was positive for ATV via PCR.

Effects on metamorphosis

Only data from metamorphosed animals were included in the following analyses. Of 160 animals, 107 (67%) metamorphosed. The percentages ranged from 60 to 75% among the eight treatments, with the exception of one treatment (larvae exposed to 0 µg/L of atrazine and $10^{3.5}$ PFU/ml of ATV), in which only 50% of animals metamorphosed. The multivariate analysis of covariance was significant for the metamorphosis-variables mass and SVL at metamorphosis and larval period (Wilks' $\lambda = 0.001$; $F = 82.911$; $df = 27, 251$; $p < 0.001$). Atrazine significantly accelerated metamorphosis ($F = 2.76$; $df = 3, 98$; $p = 0.046$) (Fig. 1a), whereas ATV had no effect on larval period ($F = 0.683$; $df = 1, 98$; $p = 0.410$). Atrazine also significantly decreased mass at metamorphosis ($F = 5.521$; $df = 3, 91$; $p = 0.002$) (Fig. 1b), whereas ATV had no effect on mass ($F = 0.029$; $df = 1, 91$; $p = 0.864$). Both atrazine ($F = 7.341$; $df = 3, 96$; $p < 0.001$) (Fig. 1c) and ATV ($F = 4.461$; $df = 1, 96$; $p = 0.037$) (Fig. 2) significantly decreased SVL at metamorphosis. Fisher's least-significant-difference test found that atrazine affected metamorphosis variables at the highest treatment level (184 µg/L) (Fig. 1).

Effects on mortality and infectivity

Atrazine alone had no appreciable effect on mortality, which is consistent with expectations resulting from our use of sublethal doses documented from other studies [22,26]. Mortality was 25% in the ATV-only treatment, which was significantly lower than the expected 50% ($\chi^2 = 5$, $df = 1$, $p = 0.025$). When the ATV-only and ATV × atrazine treatments were pooled, mortality was 16.25%, which also was significantly less than the expected 50% ($\chi^2 = 36.45$, $df = 1$, $p < 0.001$). Larvae exposed to both atrazine and ATV experienced 13.33% mortality across all three atrazine concentrations, which was significantly lower than the 25% mortality in the

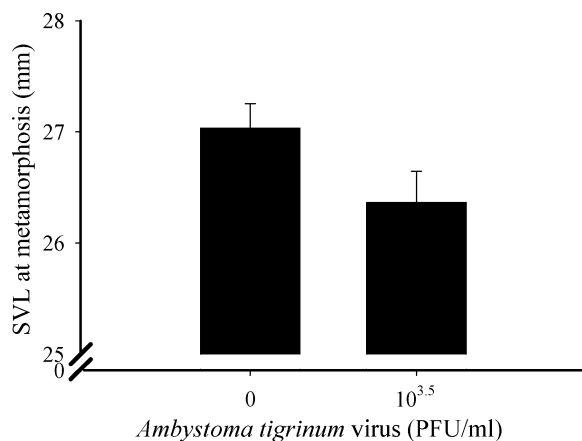


Fig. 2. Effect of *Ambystoma tigrinum* virus (ATV) on snout-vent length (SVL) at metamorphosis. Snout-vent length is significantly decreased when salamanders are exposed to ATV ($F = 4.461$; $df = 1, 96$; $p = 0.037$). Bar graphs indicate the means + standard error. PFU = plaque-forming units.

ATV-only treatment ($\chi^2 = 10.89$, $df = 1$, $p = 0.001$). The likelihood ratio (G^2) resulting from the log-linear analysis revealed a marginally significant interaction between atrazine \times virus on mortality ($G^2 = 7.75$, $df = 3$, $p = 0.052$) (Fig. 3). No pairwise differences were found among treatments (Fisher's exact test; data not shown).

No sublethal infections were found using PCR. We were unable to confirm infection in two cases of mortality (one larva exposed to 1.84 $\mu\text{g/L}$ of atrazine plus ATV and one larva exposed to 184 $\mu\text{g/L}$ of atrazine plus ATV); therefore, infection was not equal to mortality. Infectivity of ATV was significantly lower for larvae exposed to atrazine and ATV than for larvae exposed to ATV alone ($\chi^2 = 7.2$, $df = 1$, $p < 0.01$) (Fig. 4).

DISCUSSION

Transmission to alternative host

Although ATV is a virus predominantly of tiger salamanders, transmission to the northwestern salamander (*Ambyos-*

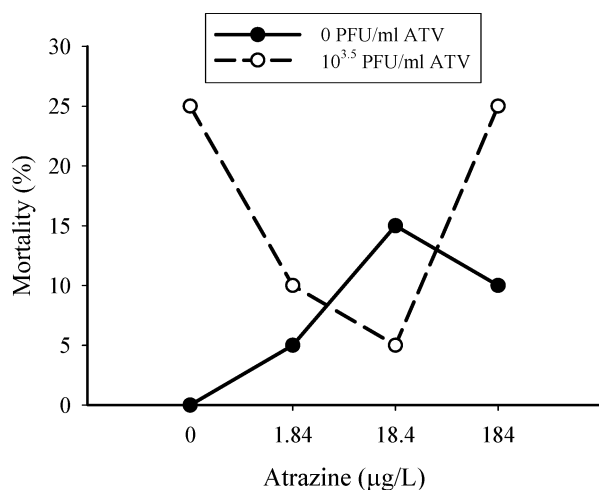


Fig. 3. Interaction plot of larval mortality \times *Ambystoma tigrinum* virus (ATV) \times atrazine ($G^2 = 7.75$, $df = 3$, $p = 0.052$). The graph suggests that when virus is absent, mortality increases at 1.84 and 18.4 $\mu\text{g/L}$ of atrazine (solid line). When virus is present, these concentrations decrease mortality (dashed line). At 184 $\mu\text{g/L}$ of atrazine, mortality is again increased in the presence of virus to percentages comparable to the virus-only treatment. However, no pairwise comparisons were significant using Fisher's exact tests. PFU = plaque forming units; G^2 = likelihood ratio.

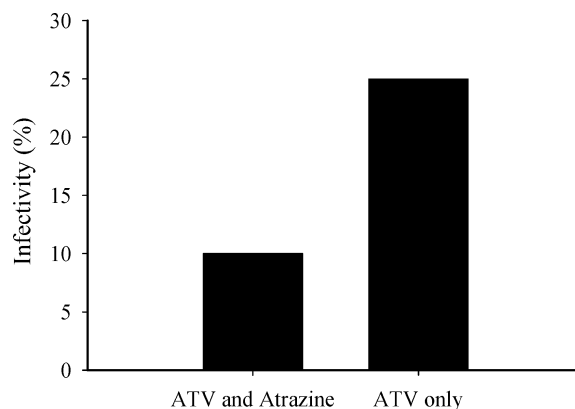


Fig. 4. Percentage infectivity of *Ambystoma tigrinum* virus (ATV) for ATV-only treatments compared to ATV and atrazine treatments. When larvae are challenged with ATV and atrazine, infectivity of ATV decreases ($\chi^2 = 7.2$, $p < 0.01$).

toma gracile) and red-spotted newt (*Notophtalmus viridescens*) has been confirmed in the laboratory [24]. We have shown that ATV also infects *A. macrodactylum*, but mortality is significantly lower than the predicted 50%. These results are consistent with the general prediction that pathogens should be locally adapted to their host [23] and that pathogen efficacy should decrease on naïve host species relative to source hosts [23]. We found no sublethally infected salamanders, suggesting that either ATV is 100% lethal to *A. macrodactylum*, or sublethal infections are cleared within 30 d (i.e., the duration of the present study). This suggests *A. macrodactylum* could be a reservoir for ATV; however, animals are unlikely to carry virus among populations or for long periods of time. More notably, populations of *A. macrodactylum* may suffer ATV epizootics if infected *A. tigrinum* and, consequently, ATV are introduced into their habitat.

Effects on metamorphosis

We found that 184 $\mu\text{g/L}$ of atrazine accelerated metamorphosis and reduced mass and SVL at metamorphosis in laboratory-exposed *A. macrodactylum* larvae. Although not studied here, the mechanism for this effect may be via an alteration of the neuroendocrine stress pathway involving the thyroid hormones and corticoid hormones [22,27]. Our findings are consistent with those from a study of the streamside salamander (*Ambystoma barbouri*), in which atrazine accelerated metamorphosis and decreased SVL and mass at metamorphosis at 400 $\mu\text{g/L}$ [28]. Atrazine also has decreased mass and SVL in *A. tigrinum* [22] as well as other anuran species [29–31]. However, studies concerning the effects of atrazine on larval period in other amphibian species have yielded either no results [26,29] or results opposite to those found in our study [30,31]. One explanation for this variation is stressor novelty. Although we did not analyze any water samples, our eggs were collected from a relatively undisturbed, high-elevation area not likely subject to agricultural pesticides beyond trace amounts left by atmospheric deposition. Nonetheless, *A. macrodactylum* have a widespread distribution throughout the Pacific and Inland Northwest that encompasses altitudes from sea level to 3,000 m [32]. Throughout its range, *A. macrodactylum* are commonly found in ponds adjacent to agricultural areas that receive direct inputs from runoff. Therefore, we chose a high-altitude pond to minimize previous atrazine exposure of embryos. A future experiment could test whether variation in larval period

resulting from atrazine exposure exists among previously exposed versus unexposed populations of *A. macrodactylum*.

Based on the present results, we believe atrazine concentrations of 184 $\mu\text{g/L}$ or greater indicate a poor aquatic environment for *A. macrodactylum*, because animals initiate metamorphosis earlier and at a smaller size when exposed to these concentrations. Larvae must reach a threshold size before they can metamorphose, but models suggest that once they reach that threshold, they may remain in the larval stage and continue to grow as long as environmental conditions are favorable or until a maximum size is obtained [33]. Larger size at metamorphosis is correlated with higher survival to maturity and reduced time to maturity, thereby increasing fitness relative to smaller metamorphosed individuals [34–36]. Smaller size at metamorphosis may be a fitness cost resulting from high-level atrazine exposure. Lighter, smaller animals have reduced terrestrial locomotor performance and, thereby, reduced ability to avoid predators or capture prey [35,36]. Smaller, newly metamorphosed adults also tend to have weakened immune systems, which could make them more susceptible to disease [3].

Furthermore, surviving ATV-treated animals had decreased SVL at metamorphosis compared to animals not challenged with ATV, although ATV had no effect on larval period or mass at metamorphosis (Fig. 2). We were not able to determine how many of our metamorphosed animals had been infected with ATV and subsequently recovered, but this result suggests that newly metamorphosed *A. macrodactylum* challenged with ATV as larvae and able to recover may metamorphose at a smaller size, thereby having potentially reduced fitness compared to uninfected animals.

Effects on mortality and infectivity

Mortality in treatment groups exposed to virus was lower than the expected 50%, and larvae exposed to both virus and atrazine together had significantly lower mortality and infectivity (Fig. 4) compared to those exposed to virus alone. Thus, atrazine may inhibit infection or ameliorate the effects of the virus, resulting in quick recovery (less than the 30-d period examined here), or atrazine may act on the immune system of the organism, somehow reducing infection. However, the underlying mechanism is unknown. It is counterintuitive that a contaminant could ameliorate the effects of a pathogen, but Parris and Baud [37] found that copper had a similar effect on the chytrid fungal pathogen *B. dendrobatidis*. Although *B. dendrobatidis* did not directly cause significant mortality in treated groups, it increased the larval period of gray tree frog (*Hyla crysoscelis*) tadpoles, a fitness cost in ephemeral habitats [38]. In treatments where doses of 64 $\mu\text{g/L}$ of copper or greater were added with *B. dendrobatidis*, the delay in larval period length was ameliorated [37].

Despite the overall decrease in mortality among animals exposed to both atrazine and virus together, differences among treatments were not as obvious. We found a marginally significant interaction of atrazine and ATV on mortality. In treatment groups exposed only to atrazine, mortality was not appreciable. However, treatment groups exposed to both atrazine and ATV appear to follow a U-shaped curve, in which low and moderate concentrations of atrazine with ATV decrease mortality compared to high concentrations of atrazine with ATV (Fig. 3). Many immunological endpoints of drugs and toxicants also follow U-shaped dose–response curves, in which smaller doses can have an immunostimulatory effect [39].

However, we are aware of no studies indicating that atrazine is an immunostimulant. Few immunological studies have looked at environmentally relevant doses of atrazine; however, the little work that has been done on amphibians suggests that atrazine acts as an immunosuppressant. Atrazine significantly decreased circulating eosinophils at 3 and 30 $\mu\text{g/L}$ in wood frogs (*R. sylvatica*) [5]. Mixtures of environmentally relevant concentrations of pesticides, which included atrazine, have been found to reduce lymphocyte proliferation in *R. pipiens* [40]. Our results suggest that atrazine levels of 20 $\mu\text{g/L}$ and lower may have an immunostimulatory effect on *A. macrodactylum*; however, immunological endpoints need to be investigated to determine actual effects of atrazine on the immune system of amphibians.

CONCLUSION

The global decline of amphibians is a complex problem with no easy solution. Multifactorial studies are essential both in understanding and disentangling the complexities of amphibian declines and in understanding the potential implications for human populations. The present study is one of the first to examine the interaction of a contaminant with ATV. The present results indicate that low to moderate atrazine concentrations may attenuate viral infection, whereas higher doses of atrazine (although still environmentally relevant) may reduce fitness by altering life-history traits in the long-toed salamander (*A. macrodactylum*). Because of species and population genetic variability, we recommend continued work on contaminant interactions with pathogens, especially in laboratory studies of currently declining amphibian populations.

Acknowledgement—The present research was funded by the National Science Foundation Division of Environmental Biology 0213851 to A. Storfer. Experimental procedures were approved by the Institutional Animal Care and Use Committee at Washington State University (Animal Subject Approval Form 3203). Special thanks are given to Andrew Giordano, Jesse Brunner, Kristen Lew, Matthew Parris, and two anonymous reviewers.

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