

ATRAZINE INCREASES RANAVIRUS SUSCEPTIBILITY IN THE TIGER SALAMANDER, *AMBYSTOMA TIGRINUM*

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Abstract. Pathogenic diseases and environmental contaminants are two of the leading hypotheses for global amphibian declines, yet few studies have examined the influence of contaminants on disease susceptibility. In this study, we examined effects of ecologically relevant doses of atrazine (0, 1.6, 16, and 160 $\mu\text{g/L}$), sodium nitrate (0, 6.8, 68 mg/L), and their interactions on susceptibility of four laboratory-bred tiger salamander families to *Ambystoma tigrinum* virus (ATV), a pathogen implicated in global amphibian die-offs. Salamanders were from Arizona populations where a coevolutionary history with ATV is supported, and thus cofactors rather than recent introduction may contribute to disease epizootics. Use of atrazine and nitrogenous fertilizers are ubiquitous; therefore, the impact of these cofactors on disease susceptibility is an important consideration. Atrazine and sodium nitrate significantly decreased peripheral leukocyte levels, suggesting an impact of these contaminants on the immune system. As expected from this result, atrazine significantly increased susceptibility of larvae to ATV infection. In contrast, nitrate had a marginally significant main effect and significantly decreased infection rate at the highest level. However, neither atrazine nor sodium nitrate had significant effects on viral copy number per individual. These results suggest that ecologically relevant concentrations of atrazine and nitrates have immunosuppressive effects, and atrazine may contribute to ATV epizootics, raising concerns about the influence of contaminants on diseases in general.

Key words: *Ambystoma tigrinum*; *Ambystoma tigrinum* virus; amphibian decline; atrazine; immune suppression; Iridovirus; Ranavirus; sodium nitrate; tiger salamander.

INTRODUCTION

Infectious diseases are increasingly recognized for their effects on species and in shaping biological communities (McCallum and Dobson 1995, Dobson and Foufopoulos 2001). Infectious diseases and environmental contaminants are two of the leading hypotheses for global amphibian declines (Collins and Storfer 2003, Storfer 2003, Stuart et al. 2004), yet very few studies have tested their interactions. The interactions of contaminants and disease may be a critical component of understanding amphibian declines, which have occurred in the past 25 (Alford et al. 2001) to 45 (Houlahan et al. 2000) years, concomitant with increased use of contaminants, such as pesticides and fertilizers. Environmental contamination may influence disease emergence by acting directly or indirectly upon the innate or adaptive immune system of the host, or by causing disruptions in homeostasis (Carey et al. 1999, Blaustein and Kiesecker 2002).

Although it has been widely hypothesized that chemically induced immunosuppression may increase host susceptibility (Daszak et al. 2001), empirical studies of the interaction of contaminants and disease are rare (Bruno et al. 2003). A notable example is an in situ

experiment that showed that simulated anthropogenic nutrient addition (of nitrate, phosphorus, and ammonium) increased the severity of coral reef diseases relative to treatments of disease or nutrients alone (Bruno et al. 2003). Another study showed that the severity of trematode (*Ribeiroia* sp.) infection, and consequently limb deformities, was increased in wood frogs (*Rana sylvatica*) in ponds adjacent to agricultural fields relative to less polluted sites (Kiesecker 2002). These studies highlight the importance of contaminant effects on disease, and the need for more empirical studies is critical because contaminant use is ubiquitous and increasing (Relyea 2005a).

Ranaviruses are one of two key pathogen groups implicated in global amphibian declines (Cunningham et al. 1996) and epizootics (Daszak et al. 2003). *Ambystoma tigrinum* virus (ATV) is responsible for epizootics in tiger salamander (*Ambystoma tigrinum*) populations throughout the western cordillera of North America from the San Rafael Valley, Arizona into Saskatchewan and Manitoba, Canada (Jancovich et al. 1997, 2005). The ATV–tiger salamander system is a model for studying wildlife diseases in general (Collins et al. 2004); however, no studies have investigated the interaction of cofactors with ATV or other ranaviruses.

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) may be an important cofactor, as it is the second most commonly used herbicide in the United

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States (Kiely et al. 2004), and is usually applied during the spring to control broadleaf weeds and grasses (Solomon et al. 1996). Atrazine is a known endocrine disruptor (Hayes et al. 2002, Hayes 2005) and a suspected immunosuppressant (Larson et al. 1998, Kiesecker 2002, Christin et al. 2003). Application of atrazine often coincides with critical amphibian larval development stages (Hayes et al. 2002), and tiger salamanders readily breed in artificial wetlands, irrigation canals, and ponds in agricultural areas (Collins 1981). Therefore, it is important and relevant to test whether atrazine influences susceptibility of tiger salamanders to ATV.

The effects on amphibians of a single contaminant are significantly altered by interactions with other contaminants (Mazanti et al. 2003, Relyea 2005a); however, studies have rarely investigated effects of multiple contaminants on disease epizootics. Nonetheless, multiple contaminants are likely present in most environments (Relyea 2005a), and atrazine is often co-applied with nitrogenous fertilizers (Kolpin et al. 1997). Eutrophication, resulting from the heavy use of nitrogenous fertilizers, can alter food webs and make conditions more favorable for pathogens (Johnson and Chase 2004, Rachowicz et al. 2005). Thus, examining atrazine and nitrate both singly and together will significantly improve our understanding of their effects on disease epizootics.

Herein, we conduct factorial experiments on laboratory-raised tiger salamander families from Arizona populations where a coevolutionary history with virus is supported (Parris et al. 2005). Thus, because it is likely that ATV and salamanders have coexisted for some time in these populations, epizootics are more likely influenced by cofactors (e.g., contaminants) than recent introduction of virus strains and a lack of host defenses. We test whether host viral infection percentage, viral copy number, and/or peripheral blood leukocyte levels are influenced by atrazine, nitrate, or their interaction. We also analyze larval period length and size (snout-vent length [SVL] and mass) at metamorphosis to determine if contaminants alter tiger salamander life-history characteristics.

MATERIALS AND METHODS

Salamander rearing

We obtained 400 eggs from four full-sibship families (100 eggs each) of laboratory-bred *Ambystoma tigrinum nebulosum* from the Arizona State University animal care facility (Tempe, Arizona, USA). We used laboratory-bred animals to eliminate the possibility of prior pathogen, atrazine, or nitrate exposure among treatment groups. Family lines originated from animals collected at locations along the Mogollon Rim (Coconino County, Arizona, USA): family MO4 from Twenty-nine Mile Lake (34°28'22" N, 111°27'15" W; elevation, 2127.4 m), family SBP from South Borrow Pit (34°28'56" N, 111°07'28" W; elevation, 2249 m), and families SO6 and

SO8 from South Tank (34°25'57" N, 110°48'51" W; elevation, 2188.4 m).

We reared eggs and larvae individually in round, polyethylene containers (12.7 × 7.62 cm) containing 500 mL of artesian spring water dechlorinated with Reptisafe (Zoo Med Laboratories, San Luis Obispo, California, USA) and aerated for at least 24 h prior to being added to experimental containers. Water was changed weekly prior to the addition of experimental treatments, and every three days thereafter. Larvae were fed 0.015 g (dry mass of eggs) of hatched brine shrimp every other day for six weeks. Beginning at six weeks of age, larvae were switched to a diet of California blackworms (*Lumbriculus variegatus*) and fed every three days ad libitum throughout the duration of the experiment. Larvae were reared in an environmental chamber on a 12:12, light:dark cycle at a constant temperature of 20° ± 1°C. All treatments were fully randomized in a fully factorial design among chamber shelves. Viral treatments were kept separately on shelves from nonviral treatments to prevent cross-contamination.

Chemical solutions

Water was changed every three days at which time we prepared fresh stock solutions of 16 mg/L atrazine (Pestanal, Sigma-Aldrich, Seelze, Germany) and 6.8 g/L sodium nitrate (NaNO₃, SigmaUltra, Sigma-Aldrich, St. Louis, Missouri, USA). Atrazine solutions were stirred vigorously on a stir plate for at least one hour to ensure formation of a homogenized solution. Sodium nitrate was stirred for only a few minutes as it readily dissolves in H₂O. Stock solutions were then diluted to make four ecologically relevant treatment concentrations of 0, 1.6, 16, and 160 µg/L atrazine (Solomon et al. 1996, Battaglin et al. 2000) and 0, 6.8, and 68 mg/L sodium nitrate (Kolpin et al. 1997, Mason 2002). Atrazine stock solution concentrations collected from two time points throughout the experiment were confirmed by liquid chromatography/gas spectrometry, and sodium nitrate concentrations collected from two time points were confirmed using ion chromatography (University of Idaho Analytical Science Laboratory, Moscow, Idaho, USA).

Experimental design

We used a complete 4 × 3 × 2 factorial design, with larvae from each of the four families randomly assigned to one of four concentrations of atrazine (0, 1.6, 16, or 160 µg/L), one of three concentrations of sodium nitrate (0, 6.8, or 68 mg/L), and either none or 1 × 10⁴ plaque forming units (pfu)/mL ATV. Individuals were the unit of replication, and thus a total of 384 larvae were used (16 per treatment, four from each family). The ATV isolate was extracted from a larval *A. tigrinum nebulosum* collected at a die-off site along the North Rim of the Grand Canyon (Coconino County, Arizona, USA), the geographically closest viral strain to the source of the salamanders used herein (Mogollon Rim, Arizona).

Larvae were exposed to experimental treatments at 12 weeks of age. This age was chosen because ambystomatid salamanders are unable to form immunoglobulins before this age (Fellah et al. 1992), and peak epizootics seem to occur around this age in nature (Brunner et al. 2004). Larvae in viral treatments were exposed to 1×10^4 pfu/mL ATV for three days, sufficient to induce infection in previous experiments (Jancovich et al. 2001, Brunner et al. 2004). The first water change occurred four days after initial introduction of atrazine due to a 24-h temperature irregularity (that was subsequently repaired) in the environmental chamber that prevented viral introduction until day two. Water pH did not change after three days; therefore, it is unlikely that four days had an appreciable effect on atrazine concentrations. All subsequent water changes occurred every three days to prevent degradation of atrazine, which has a half-life of three to 90 days in water (Solomon et al. 1996). As acidic pH can accelerate degradation (Solomon et al. 1996), all larvae were maintained in pH between 7.0 and 8.0, the pH of the spring water used in the experiment. Nitrates are highly stable in aquatic environments without vegetation to convert them to nitrogen gas (Camargo and Ward 1992); therefore, it is unlikely that nitrate concentrations decreased appreciably within each three-day period.

Mass (to the nearest 0.05 g) measurements were taken during each water change, and both mass and SVL measurements (to the nearest 0.1 mm) were taken at metamorphosis. Upon metamorphosis, a portion of the tail was clipped and a drop of blood was collected from the caudal vein. A blood smear was prepared for leukocyte analysis. The tail clip was preserved in 95% ethanol and stored at -20°C for DNA extraction and viral detection via PCR (polymerase chain reaction). The metamorphosed salamander was then euthanized according to IACUC (Institutional Animal Care and Use Committee)-approved protocols. Blood was collected prior to euthanasia to prevent confounding treatment-related effects on leukocyte counts with death-related effects. Other laboratory studies have determined that most viral-related deaths occur between 7 and 21 d postviral exposure (Jancovich et al. 1997, 2001, Brunner et al. 2004; D. D. Forson, *unpublished data*) at similar temperatures (Rojas et al. 2005). Therefore, salamanders that metamorphosed prior to 23 d were not processed and euthanized until 23 d to allow sufficient time for any viral-related deaths to occur. No animals died after day 20 postexposure or showed symptoms of infection, which usually occur 72 hours prior to death (D. D. Forson and A. Storfer, *unpublished data*). After 23 d, metamorphosed salamanders were processed upon metamorphosis. The experiment ended after no animal had metamorphosed for 14 days (at 111 d). Note that tiger salamanders are often neotenic (i.e., becoming sexually mature in the larval form) and thus, this was a reasonable time to end the experiment. These remaining 30 non-metamorphosed larvae were euthanized. An

additional 31 animals died before metamorphosis, and, thus a total of 61 animals were omitted from the larval period and size at metamorphosis analyses.

Real-time PCR

We used real-time PCR to identify whether individuals were infected and to quantify viral levels per infected individual to compare severity of infection among treatments. We extracted DNA from tail clip tissue (sufficient to detect sublethal infection in previous experiments; Brunner et al. 2004; A. Storfer, *unpublished data*) using either DNAeasy 96 tissue kits (Qiagen, Valencia, California, USA) or a standard phenol/chloroform protocol. (Note that DNA quality did not differ between the two methods of extraction.) A 70 base-pair region of the major capsid protein of ATV was amplified using MCP forward primer (5' ACA CCA CCG CCC AAA AGT AC 3') and MCP reverse primer (5' CCG TTC ATG ATG CGG ATA ATG 3') as well as a fluorescent probe (5' FAM-CCT CAT CGT TCT GGC CAT CAA CCA C-TAM 3') (Brunner et al. 2004). The PCR reaction included 100-ng DNA template standardized among all samples using a VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, California, USA), 300 nmol forward primer, 900 nmol reverse primer, 250 nmol probe, and Taqman 2X Universal PCR Master Mix (no AmpErase UNG)(Applied Biosystems, Foster City, California, USA).

We ran PCR reactions on an ABI 7300 Real-time PCR System using Real-time PCR System Sequence Detection Software version 1.2.3 (Applied Biosystems, Foster City, California, USA) for 40 cycles: 95°C denaturing (20 s), 54°C annealing (20 s), and 72°C extension (30 s). The viral isolate used to infect larvae was also used to set up a standard curve for absolute quantification. Viral DNA for the standard curve was extracted from pure EPC culture using a modified Hirt (1967) procedure (see Jancovich et al. 2005) and quantified. We prepared a log-based dilution series of 5×10^7 viral copies/ μL to 5×10^3 viral copies/ μL for the standard curve based on the known ATV genome size (see Jancovich et al. 2005). Standard curve samples were run in triplicate and unknowns were run in duplicate. We manually set the CT (threshold cycle) at 1.0 in the amplification range for all runs, and duplicate samples with a coefficient of variation (cv) $>20\%$ were rerun in duplicate until the cv was $<20\%$. All animals in the control treatment (i.e., those exposed to 0 $\mu\text{g/L}$ atrazine, 0 mg/L sodium nitrate, and 0 pfu/mL ATV) were screened for virus, as were 25% of larvae from all other treatments exposed to 0 pfu/mL ATV. These larvae were all negative for ATV DNA and were not used in statistical analyses comparing ATV infection levels among atrazine and nitrate treatments.

Peripheral blood leukocytes

We counted the number of peripheral blood leukocytes per individual blood smear to compare immune

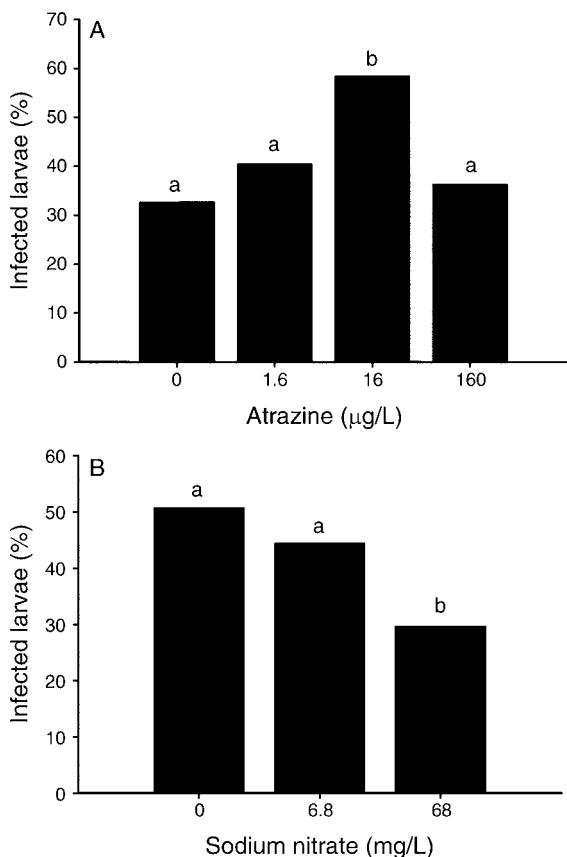


FIG. 1. Effect of atrazine ($G^2 = 52.35$, $df = 36$, $P = 0.038$) and sodium nitrate ($G^2 = 45.77$, $df = 32$, $P = 0.054$) on viral infection rate. (A) The number of ATV-infected larvae is higher at 16 µg/L atrazine than at the other atrazine treatments levels. (B) The number of ATV-infected larvae is lower at the highest treatment level of 68 mg/L compared to the other treatments. Different letters represent significantly different comparisons ($P < 0.05$).

system activity among treatments. Blood smears were prepared using blood collected from the caudal vein, and the smears were stained with Wright-Giemsa stain (Sigma-Aldrich, St. Louis, Missouri, USA). Blood smears were examined under a compound light microscope at 400× magnification. A high quality portion of the smear containing a consistent distribution of red blood cells was chosen (i.e., not containing clumps of red blood cells or gaps with no red blood cells). Because standardization of the fields was not exact, we averaged total number of leukocytes across 10 fields for each slide. Fields were selected without bias in a straight-line or rectangular pattern based on smear quality.

Statistical analysis

We analyzed data using Systat 11 (Systat Software, Point Richmond, California, USA) and SAS 9.0 (SAS Institute, Cary, North Carolina, USA) software. We tested the effects of atrazine, nitrate, ATV, and their interactions on metamorphosis (larval period, mass, and

SVL), viral infection, and leukocyte level. Mixed-model analyses of covariance (ANCOVAs), with family as a random effect and initial mass (mass measured on day one of experiment, just prior to treatment addition) as a covariate, were used to examine differences in viral copy number, average leukocyte count, larval period, and size (mass and SVL) at metamorphosis among treatments. Analysis of viral copy number included only larvae exposed to virus because unexposed larvae were negative for ATV DNA, while analysis of leukocyte counts and metamorphosis variables included all larvae. Because larval period and size at metamorphosis are correlated, we analyzed these response variables using multivariate analysis of covariance (MANCOVA), with mass prior to exposure as a covariate, before analyzing univariate effects. Frequency distributions of viral copy number and leukocyte count were both right-skewed; therefore, the natural log of viral copy number and the square root of the leukocyte count were used to normalize data to meet assumptions of ANOVA models. Fisher's least significant difference tests were used for multiple comparisons among treatment levels. Effects of treatments on infection rates were analyzed using log-linear analysis, with comparisons among treatments analyzed using chi-square analysis.

RESULTS

Disease susceptibility

Atrazine caused significant differences in infection rates among treatments ($G^2 = 52.35$, $df = 36$, $P = 0.038$), and sodium nitrate had a marginally significant effect ($G^2 = 45.77$, $df = 32$, $P = 0.054$). Infection rates were significantly higher at the moderate concentration of 16 µg/L atrazine compared to all other concentrations tested (Fig. 1A). The highest concentration of atrazine tested (160 µg/L) did not affect infection rates relative to controls (Fig. 1A); however, the highest nitrate concentration tested (68 mg/L) significantly lowered infection rates (Fig. 1B). Atrazine significantly lowered leukocyte counts at 16 µg/L and 160 µg/L atrazine compared to 0 µg/L and 1.6 µg/L (Table 1, Fig. 2A). Nitrate also affected leukocyte count (Table 1), with both significantly lower circulating leukocyte levels at 6.8 mg/L and 68 mg/L sodium nitrate compared to 0 mg/L nitrate (Fig. 2B). There were family ($\gamma = 1.438$) and family by atrazine ($\gamma = 2.463$) interaction effects that obscured the main effects of atrazine on infection level (i.e., viral copy number; Table 1, Fig. 3). Nitrate did not have significant effects on viral copy number, nor was there a nitrate by atrazine interaction (Table 1). No control animals were infected.

Metamorphosis

The overall MANCOVA for mass and SVL at metamorphosis and larval period was significant (Wilks' lambda = 0.001, $F_{264, 657} = 20.019$, $P < 0.001$). The only treatment that affected mass at metamorphosis was virus (Table 2), with exposure to 1×10^4 pfu/mL ATV

TABLE 1. Statistical summary of mixed-model ANCOVA results for treatment main effects and their interactions on viral copy number and leukocyte count.

Response variable and source of variation	df	F	P
Viral copies			
Initial mass (covariate)	1, 141	2.35	0.127
Atrazine	3, 9	2.86	0.097
Nitrate	2, 6	1.94	0.224
Atrazine × nitrate	6, 18	0.40	0.868
Leukocyte count			
Initial mass (covariate)	1, 280	0.68	0.411
Atrazine	3, 280	5.03	0.002
Nitrate	2, 280	5.96	0.003
Virus	1, 280	0.68	0.411
Atrazine × nitrate	6, 280	0.49	0.813
Atrazine × virus	3, 280	0.41	0.747
Nitrate × virus	2, 280	1.55	0.214
Atrazine × nitrate × virus	6, 280	0.87	0.520

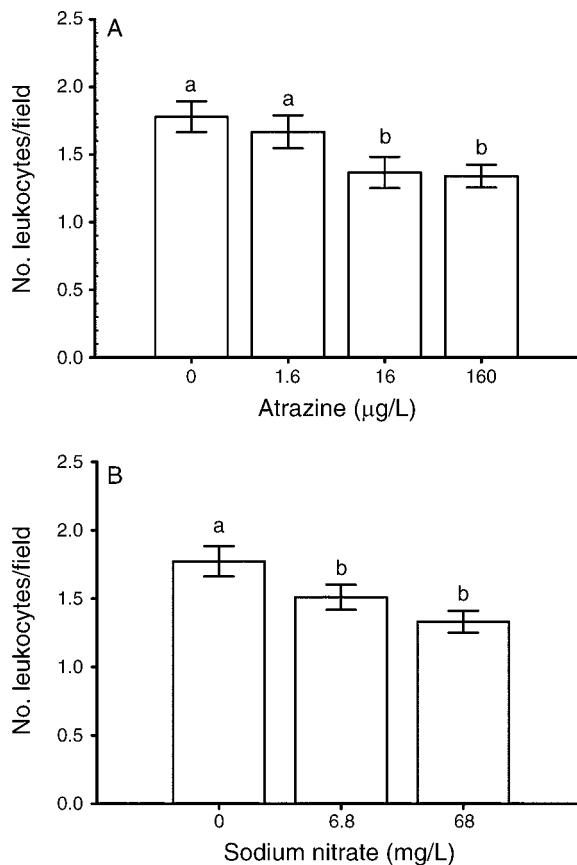


FIG. 2. Effects of atrazine ($F_{3,280} = 5.03, P = 0.002$) and sodium nitrate ($F_{2,280} = 5.96, P = 0.003$) on the number (mean \pm SE) of peripheral leukocytes. (A) Leukocyte counts are lower at 16 $\mu\text{g/L}$ and 160 $\mu\text{g/L}$ compared to 0 $\mu\text{g/L}$ and 1.6 $\mu\text{g/L}$. (B) Leukocyte counts are lower at 6.8 and 68 mg/L sodium nitrate compared to 0 $\mu\text{g/L}$. Different letters represent significantly different comparisons ($P < 0.05$).

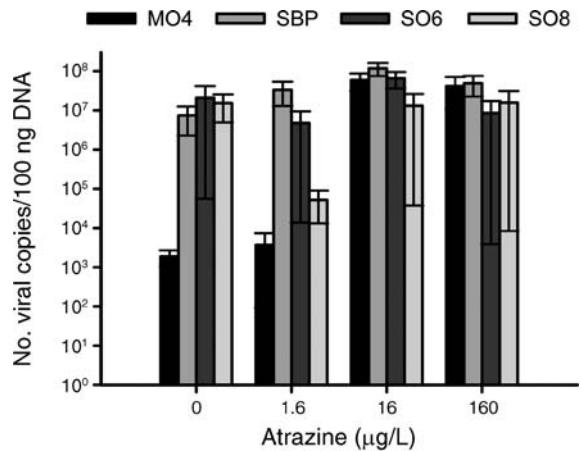


FIG. 3. Effects of family on viral copy number per salamander at each atrazine treatment level ($\gamma = 2.463$). Note the log scale. There were no significant differences among atrazine treatments on viral copy number per salamander. However, there was significant family variation at 0 $\mu\text{g/L}$ and 1.6 $\mu\text{g/L}$ atrazine for viral copy number, largely due to family MO4.

slightly increasing mass relative to uninfected controls. No treatments significantly affected SVL at metamorphosis. Atrazine had a marginally significant effect on larval period (Table 2), with 16 $\mu\text{g/L}$ atrazine accelerating metamorphosis compared to 1.6 $\mu\text{g/L}$, but not 0 $\mu\text{g/L}$ atrazine.

TABLE 2. Statistical summary of mixed-model ANCOVA results for treatment main effects and their interactions on mass and SVL and larval period at metamorphosis.

Response variable and source of variation	df	F	P
Mass			
Initial mass (covariate)	1, 291	0.52	0.472
Atrazine	3, 291	1.73	0.160
Nitrate	2, 291	0.42	0.657
Virus	1, 291	3.92	0.049
Atrazine × nitrate	6, 291	0.90	0.497
Atrazine × virus	3, 291	0.17	0.916
Nitrate × virus	2, 291	0.76	0.468
Atrazine × nitrate × virus	6, 291	1.15	0.332
SVL			
Initial mass (covariate)	1, 283	1.23	0.268
Atrazine	3, 283	1.74	0.160
Nitrate	2, 283	0.45	0.641
Virus	1, 283	0.92	0.339
Atrazine × nitrate	6, 283	1.18	0.319
Atrazine × virus	3, 283	0.50	0.683
Nitrate × virus	2, 283	0.55	0.575
Atrazine × nitrate × virus	6, 283	0.86	0.523
Larval period			
Initial mass (covariate)	1, 291	0.08	0.773
Atrazine	3, 291	2.45	0.063
Nitrate	2, 291	1.77	0.171
Virus	1, 291	0.27	0.601
Atrazine × nitrate	6, 291	1.58	0.153
Atrazine × virus	3, 291	0.28	0.841
Nitrate × virus	2, 291	0.64	0.530
Atrazine × nitrate × virus	6, 291	0.58	0.746

DISCUSSION

Disease susceptibility

Intermediate, ecologically relevant concentrations of atrazine resulted in significantly increased infection rates (Fig. 1A), but a family by atrazine interaction obscured the main effect of atrazine on viral copies per individual. In tiger salamanders, these results may indicate that contaminant exposure increases the proportion of the population infected by ATV. Because viral dynamics are generally thought to be density dependent rather than dependent on viral load per larvae (Brunner et al. 2004), our results indicate that contaminant exposure may increase the severity of epizootics by increasing the rate of infection. High, but ecologically relevant, concentrations of nitrates may decrease infection rates possibly by directly killing ATV; however the mechanism is unknown and the main effect of nitrate was marginally significant ($P = 0.054$) in our study (Fig. 1B). Leukocyte levels were significantly decreased by both atrazine (Fig. 2A) and sodium nitrate (Fig. 2B), suggesting that these contaminants may result in immunosuppression. These results are an important step in our understanding of disease emergence in amphibians. Few studies have tested the interaction of environmental contaminants and pathogens, two leading hypotheses for amphibian declines. Atrazine, in particular, is the most widely used herbicide worldwide (Hayes et al. 2002), and thus concern should be raised regarding the potential effects of atrazine or combinations with other stressors on disease emergence in amphibians and in general. Multifactorial experiments have generally provided great insights into effects of combined stressors on amphibians, such as synergistic effects of exposure to pesticides and predators that are generally more lethal than exposure to either stressor alone (Relyea and Mills 2001, Relyea 2004, 2005b).

Environmental stress generally results in increased production of corticotropin-releasing hormone, increasing the overall levels of circulating T_3 , T_4 , and corticosterone (Denver 1997), which may decrease leukocyte activity (Saad et al. 1987). Atrazine and pesticide mixtures containing atrazine have been shown to reduce lymphocyte proliferation in leopard frogs (*Rana pipiens*; Christin et al. 2003) and circulating eosinophils in wood frogs (*Rana sylvatica*; Kiesecker 2002). Although we did not test the mechanism, it is possible that concentrations of 16 $\mu\text{g/L}$ atrazine result in a stress response, which increases corticotropin-releasing hormone, leading to the observed decrease in peripheral leukocytes and an increase in susceptibility to viral infection. Future study could examine this mechanism directly by testing blood hormone levels.

In a previous study, we found that intermediate levels (1.84 $\mu\text{g/L}$ and 18.4 $\mu\text{g/L}$) of atrazine decreased ATV infection relative to controls and the highest level (184 $\mu\text{g/L}$) of atrazine in ATV-exposed long-toed salamanders (*Ambystoma macrodactylum*; Forson and Storfer

2006). It is unclear why moderate concentrations of atrazine had an immunostimulatory effect on *A. macrodactylum* and an apparent immunosuppressive effect on *A. tigrinum*. Differences in experimental design, such as the use of commercial grade atrazine rather than technical grade in the *A. macrodactylum* experiment, or that long-toed salamanders are not the source host for ATV, may account for these contradictory results.

Although we found no significant main effects of atrazine on the number of viral copies per individual, there was a significant atrazine \times family interaction (Fig. 3). Two of the families (SBP and S06) had high viral loads regardless of atrazine concentration, while the other two families varied in their viral load. This result may be ecologically important because viral load and transmission are usually correlated (Ewald 1994). Recent work suggests that it is relatively common to have some individuals in a population act as "super-spreaders" by having significantly higher transmission rates than other individuals (Lloyd-Smith et al. 2005). Usually, the capacity of a pathogen to spread is calculated by estimating the basic reproductive number of epidemic spread (R_0), or the mean number of individuals infected per infected individual. Calculating a mean value such as R_0 can obscure individual variation in infectiousness. However, individual super-spreaders can have a significant effect on probability of disease emergence; for example, a single individual was primarily responsible for the SARS outbreak (Lloyd-Smith et al. 2005). In addition, single population/family studies may not reflect natural variation in responses to disease and contaminants. For example, Bridges and Semlitsch (2000, 2001) found significant differences in tolerance to carbaryl between *Rana* spp., and within populations and among full-sib families of the southern leopard frog (*Rana sphenoccephala*). Thus, our results highlight the need for incorporating individual and family-level variation in disease emergence studies.

Overall, emergence of ATV in tiger salamanders is likely due to a combination of both human introduction and other anthropogenic factors, such as pollution. Phylogeographic analyses suggest recent spread of ATV among western North American tiger salamander populations, possibly through anthropogenic movement of infected salamanders used as fishing bait (Jancovich et al. 2005). Nonetheless, ATV is also likely endemic to parts of Arizona because a coevolutionary history of tiger salamanders and ATV is supported via an inverse correlation between disease frequency and frequency of cannibalistic salamanders throughout Arizona, likely driven by past selection (Pfennig et al. 1991, Parris et al. 2005). In these areas, environmental contaminants such as atrazine may contribute to disease emergence or increased severity of epizootics.

Metamorphosis

Contaminants may induce a stress response in amphibians, increasing circulating corticoid levels and

leading to a decreased larval period (Denver 1997, Hayes 1997). We found no main effect of atrazine or nitrate on larval period, or size at metamorphosis (Table 2). However, other studies on ambystomatid salamanders have shown decreases in size at metamorphosis at exposures above 184 µg/L (Larson et al. 1998, Rohr et al. 2004, Forson and Storfer 2006). Nitrate did not affect larval period or size at metamorphosis (Table 2), yet nitrate concentrations similar to those tested herein have been found to decrease SVL and growth rates in other amphibian species (Allran and Karasov 2000, Sullivan and Spence 2003). These conflicting results suggest there may be species differences in their response to atrazine, nitrates, and virus.

Perhaps surprisingly, ATV caused a slight, but significant increase in mass at metamorphosis (Table 2). This slight increase in mass is likely due to edema, a symptom of ATV infection (Bollinger et al. 1999; D. D. Forson, unpublished data). ATV has caused decreases in SVL in other studies (Parris et al. 2005, Forson and Storfer 2006), but we found no significant difference herein.

In sum, our study suggests that atrazine could be a factor in the emergence of ATV in western North America and that nitrate may compromise immunity by decreasing leukocyte levels. Although studies of the interactions of contaminants and diseases are rare, the few studies that do exist suggest contaminants can increase disease severity (Kiesecker 2002, Bruno et al. 2003), potentially by compromising immunity (Kiesecker 2002). Amphibians are considered to be indicators of environmental quality because of their permeable skin and biphasic life history (Blaustein and Kiesecker 2002); therefore, adverse effects of environmentally relevant concentrations of pesticides are of concern for all biota.

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