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Original Contribution

Combined Effects of Atrazine and Chlorpyrifos on Susceptibility of the Tiger Salamander to *Ambystoma tigrinum* Virus

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Abstract: Several hypotheses have been examined as potential causes of global amphibian declines, including emerging infectious diseases and environmental contaminants. Although these factors are typically studied separately, animals are generally exposed to both stressors simultaneously. We examined the effects of the herbicide atrazine and the insecticide chlorpyrifos on the susceptibility of tiger salamander larvae, *Ambystoma tigrinum*, to a viral pathogen, *Ambystoma tigrinum* virus (ATV). Environmentally relevant concentrations of atrazine (0, 20, 200 μg/L) and chlorpyrifos (0, 2, 20, 200 μg/L) were used along with ATV in a fully factorial experimental design whereby individually housed, 4-week-old larvae were exposed for 2 weeks. Atrazine alone was not lethal to larvae, and chlorpyrifos alone was lethal only at the highest concentration. When combined with ATV, chlorpyrifos increased susceptibility to viral infection and resulted in increased larval mortality. A significant interactive effect between atrazine and ATV was detected. Atrazine treatments slightly decreased survival in virus-exposed treatments, yet slightly increased survival in the virus-free treatments. These findings corroborate earlier research on the impacts of atrazine, in particular, on disease susceptibility, but exhibit greater effects (i.e., reduced survival) when younger larvae were examined. This study is the first of its kind to demonstrate decreases in amphibian survival with the combination of pesticide and a viral disease. Further examination of these multiple stressors can provide key insights into potential significance of environmental cofactors, such as pesticides, in disease dynamics.

Keywords: pesticide, disease, multiple stressors, amphibian, salamander, virus

INTRODUCTION

Amphibian declines are occurring worldwide due to a variety of anthropogenic impacts (Blaustein and Kiesecker, 2002). Whereas clear mechanisms likely underlie many of these declines (e.g., habitat loss and invasive species), other causes are far less understood and can involve subtle and complex interactions (Collins and Storfer, 2003). These ''enigmatic'' declines account for a large proportion of worldwide population losses and require further empirical and field-based investigation (Stuart et al., 2004). Two primary suspects for these enigmatic declines are emerging infectious diseases and contaminant exposure.

Disease is thought to play a key role in amphibian declines worldwide (Daszak et al., 2003; Stuart et al., 2004). Several diseases have been implicated in these declines:

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ranaviral diseases, chytridiomycosis, saprolegniosis, and *Ribeiroia* infection (Carey, 1993; Berger et al., 1998; Daszak et al., 1999; Kiesecker, 2002; Johnson et al., 2007). In particular, Ranaviruses and the chytrid fungus, *Batrachochytrium dendrobatidis*, are well-documented for causing population die-offs throughout the world (Daszak et al., 1999, 2003). What is far less clear about both of these pathogens is the reason(s) for their apparent sudden emergence (Chinchar, 2002; Daszak et al., 2003; Collins and Halliday, 2005). One proposed mechanism for this emergence is that exposure to contaminants can weaken host immune responses and increase susceptibility to infection and disease (Carey, 2000; Christin et al., 2003; Forson and Storfer, 2006a; Rohr et al., 2008).

Several pesticides have been shown to exhibit strong negative impacts on amphibians. Chlorpyrifos is the most widely used insecticide in the United States and, although targeted to act on insect nervous systems (Kiely et al., 2004), it also is known to alter nervous function in amphibians (Colombo et al., 2005). These effects result in both behavioral modifications and a reduction in growth (Widder and Bidwell, 2006). Chlorpyrifos also has a large range of lethal concentration (LC_{50}) values ranging from 1 to 3,000 μg/L for differing amphibian species (Barron and Woodburn, 1995). It is estimated that concentrations up to 64 μg/L occur in natural settings (van den Brink et al., 1996), with residues in natural ponds found at >20 μg/L (Hurlbert et al., 1970).

Atrazine is one of the most widely used herbicides across the United States and is frequently detected in a wide variety of water bodies at concentrations up to 250 μg/L (Solomon et al., 1996). Atrazine has been shown to disrupt endocrine function in two species of frogs (Hayes et al., 2002, 2003). Atrazine has other large negative effects in several amphibian species in terms of growth inhibition, developmental suppression, and decreased survival (e.g., Larson et al., 1998; Diana et al., 2000; Storrs and Kiesecker, 2004; Rohr et al., 2006). In addition, atrazine has been found to elicit negative effects indirectly via depleting amphibian food resources (Boone and James, 2003) and by increasing amphibian susceptibility to trematode infection (Kiesecker, 2002; Rohr et al., 2008).

Tiger salamanders (*Ambystoma tigrinum*), like many other amphibian species, have undergone widespread epizootics throughout the western United States (Jancovich et al., 1997, 2005). A major cause for these die-offs has been the emergence of a single host ranaviral pathogen, *Ambystoma tigrinum virus* (ATV) (Jancovich et al., 1997, 2005; Storfer et al., 2007). It is hypothesized that environmental cofactors might have triggered some of these recent outbreaks (Jancovich et al., 2005; Forson and Storfer, 2006a, b).

There is some evidence that pesticides could be important cofactors for amphibian declines in general. Pesticide contamination occurs throughout the United States, from agricultural ponds to relatively undisturbed sites in the Sierra Nevada mountains of California (McConnell et al., 1998). The concentrations detected at most of these sites are typically too low to cause mortality directly, even among amphibian larvae. The sublethal effects of these low concentrations, and in particular, their interaction with biotic stressors are not well-understood. One study performed on 12-week-old *A. tigrinum* larvae showed increased infection rates with ATV when also exposed to atrazine at 16 μg/L (Forson and Storfer, 2006a). Only until recently, have the potential interactive effects of pesticides and pathogens in amphibians been explored (Kiesecker, 2002; Christin et al., 2003).

In addition to interactions with pathogens, recent studies have found interactive effects of sublethal concentrations of atrazine with other pesticides. Belden and Lydy (2000) found that atrazine potentiates the toxicity of chlorpyrifos in chironomid midges. When sublethal concentrations of each pesticide are combined, midge survival is significantly reduced. This potentiating effect has shown mixed results among amphibian taxa. The pesticide combination results in lethality in the African clawed frog, *Xenopus laevis,* but not in the wood frog, *Rana sylvatica,* or the green frog, *Rana clamitans* (Wacksman et al., 2006). Combined effects of atrazine and chlorpyrifos have not been yet examined in salamander taxa, but may have important implications for disease dynamics because the two pesticides often are applied at the same sites (Wacksman et al., 2006).

In this study, we examine the effects of the multiple stressors of a viral pathogen (ATV) and the pesticides atrazine and chlorpyrifos on 4-week-old tiger salamander larvae. We also examine interactions between the two pesticides, at several ecologically relevant concentrations, with one another and ATV in a fully factorial experimental design. We hypothesize that the presence of pesticides increases susceptibility to ATV and results in reduced survival of larval salamanders.

Methods

Salamander Larvae

We used laboratory-bred larvae to ensure no previous exposure to the pathogen or pesticides. Eggs were obtained from five full sibship families at Arizona State University (Tempe, AZ, USA). Families originated from animals collected along the Mogollon Rim (Coconino County, AZ, USA). Larvae were reared individually in round polyethylene containers (12.7-cm × 7.6-cm) filled with 500 ml of artesian spring water treated with Reptisafe (Zoo Med Laboratories, San Luis Obispo, CA, USA), which was aerated for at least 24 hours. Complete water changes were performed weekly both during the rearing period and the experiment. Upon hatching, larvae were fed daily 0.015 g of egg dry mass of hatched brine shrimp. Larvae were maintained on a 12:12 hour light:dark cycle in an environmental chamber kept at 20 ± 1°C. The experiment began once larvae reached 4 weeks of age (mass = 0.10 ± 0.0018 g; SVL = 0.88 ± 0.21 cm).

Experimental Design

A fully factorial 3 (0, 20, 200 µg/L atrazine) × 4 (0, 2, 20, 200 µg/L chlorpyrifos) × 2 (virus/no virus) design was used, replicating each treatment 10 times with individual animals as replicates, for a total of 240 animals. Individuals from each of the five families were distributed equally to all treatments. Larvae were individually housed in plastic cups filled with 500 ml of well water. Pesticide aliquots were administered first, with virus aliquots immediately following. Using a previous protocol (Forson and Storfer, 2006a), we administered to each individual cup an aliquot of control cell media or media containing the viral strain CAP, which originates from the same population that our experimental salamanders were drawn from (Kaibab region, Arizona). The viral strain used was passed through cell culture only twice, and therefore was suitable for use. Larvae in the viral treatment were exposed to 1×10^4 plaque forming units of *Ambystoma tigrinum* virus via water bath for 7 days (estimated LC_{50}; Brunner et al., 2005). Pesticides were reapplied after the water change on day 7, whereas virus exposure occurred only during the initial week. The experiment was concluded after 2 weeks, when death had subsided in virus treatments for 3 consecutive days. Surviving larvae were killed at the end of the experiment with a water bath overdose of MS-222.

Pesticide Preparation

For initial exposure and each subsequent water change, a new 100-mg/L stock solution was created for each pesticide. Technical grade atrazine and chlorpyrifos were dissolved in 100% methanol and then subsequently diluted 10x into distilled water to create working solutions. These solutions were vigorously shaken for 10 minutes to ensure homogenization. From this, 100 and 1,000 µl of solutions were placed in their respective treatments of 500 ml of water to obtain 20 and 200 µg/L concentrations of each pesticide. For chlorpyrifos, a 2 µg/L concentration also was added by using 10 µl of the stock solution. Control treatments were administered: 1000 µl of a 10% methanol solvent control. Stock solution concentrations of both pesticides were verified via gas chromatography (University of Idaho Analytical Science Laboratory, Moscow, ID, USA). Previous research shows negligible degradation of both pesticides during a 7-day period in laboratory setups (Manzanti et al., 2003).

Variables Measured

Survival of individuals was monitored daily, with deceased organisms immediately preserved in 95% ethanol. To quantify virus loads and infection status of individuals, we used quantitative real-time PCR. Methodology of DNA extraction and qPCR followed Forson and Storfer (2006a). Tail tissue from larvae were extracted via DNeasy kits (Qiagen) and quantified via a Nanodrop spectrophotometer. Samples were diluted to 20-ng/L concentrations and examined in triplicate. Reactions contained 100-ng template DNA, 300-nmol forward primer, 900-nmol reverse primer, 240-nmol probe, and Taqman 2X Universal PCR master mix (no AmpErase UNG; Applied Biosystems, Foster City, CA, USA). Reactions were run for 40 cycles of 95°C denaturing (20 s), 54°C annealing (20 s), and 72°C extension (30 s) on an ABI 7300 Real-time PCR System using Real-time PCR System Sequence Detection Software version 1.2.3 (Applied Biosystems). All virus-treated animals were examined along with a random sampling of 20% of the no-virus controls to verify lack of contamination.

Statistical Analyses

We used logistic regressions to detect significant interactions between treatments of virus, atrazine, and chlorpyrifos using the response variables of both total dead and infected

(PROC LOGISTIC, SAS 9.0). The total number of viral copies data were log transformed (Log (x + 1)) to meet normality assumptions. We analyzed these transformed data using two-way ANOVA. Due to the bimodal nature of the data, we also statistically examined differences in viral load between non-zero values (i.e., only infected individuals).

Results

Mortality

The logistic regression analysis listed three key components to the overall model: virus, chlorpyrifos, and a virus × atrazine interaction. Virus exposed treatments resulted in significantly higher mortality across all treatments relative to nonvirus treatments ($\chi^2 = 40.59$, degrees of freedom (df) = 1, $p = 0.0001$). Significant increase in mortality due to pesticide exposure was detected with chlorpyrifos ($\chi^2 = 16.88$, df = 3, $p = 0.0007$), with increasing concentrations resulting in higher mortality regardless of virus presence (Figure 1). The combination of virus and chlorpyrifos resulted in an additive increase in mortality relative to the pesticide alone, although the interaction term was not significant ($\chi^2 = 1.88$, df = 3, $p = 0.6$). We found no significant main effect of atrazine on mortality ($\chi^2 = 2.46$, df = 2, $p = 0.29$) and no significant interactive effects of atrazine by chlorpyrifos on mortality ($\chi^2 = 7.59$, df = 6, $p = 0.27$). There was a significant interactive effect of atrazine by virus ($\chi^2 = 6.68$, df = 2, $p = 0.03$) with the combination of atrazine slightly reducing mortality in no-virus treatments and slightly increasing mortality in virus treatments (Figure 2). The three-way interaction effect between virus, chlorpyrifos, and atrazine was not significant ($\chi^2 = 8.36$, df = 6, $p = 0.21$), although combined pesticide treatments resulted in the greatest mortality in virus-exposed animals (Figure 3).

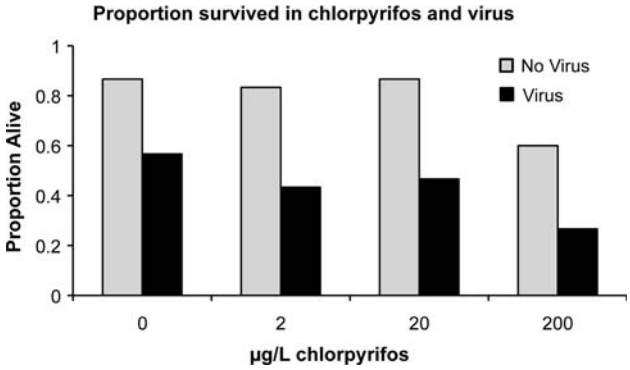

Figure 1. Proportion of salamander larvae that survived viral and chlorpyrifos exposure. Pesticide levels span ecologically relevant concentrations and viral exposure level typically results in 50% survival. Logistic regression analyses are performed on total proportions, and therefore no standard error bars are present. Each bar represents all larvae in a particular treatment, and therefore combines larvae across all atrazine treatments.

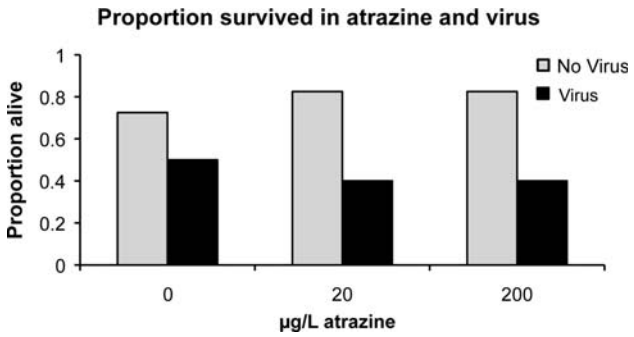

Figure 2. Proportion of salamander larvae that survived viral and atrazine exposure. Pesticide levels span ecologically relevant concentrations and viral exposure level typically results in 50% survival. Logistic regression analyses are performed on total proportions, and therefore no standard error bars are present. Each bar represents all larvae in a particular treatment, and therefore combines larvae across all chlorpyrifos treatments.

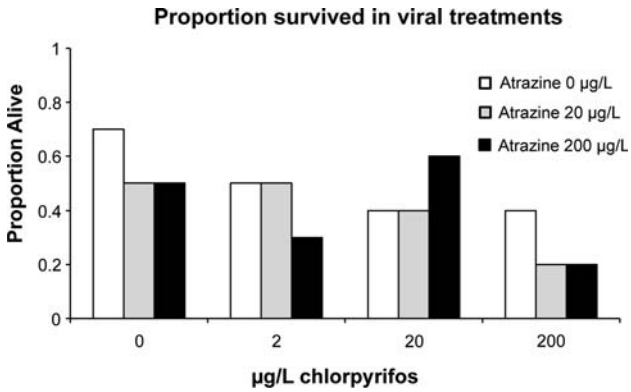

Figure 3. Proportion of salamander larvae that survived viral and pesticide exposure. Virus only data presented to show additive effects of increasing pesticide concentrations. Logistic regression analyses are performed on total proportions, and there no standard error bars are present.

Infection

The logistic regression model showed no interactive effect between the two pesticides on infection rate was detected ($\chi^2 = 6.33$, $p = 0.85$). When examined separately, increased chlorpyrifos concentration resulted in higher infection rates

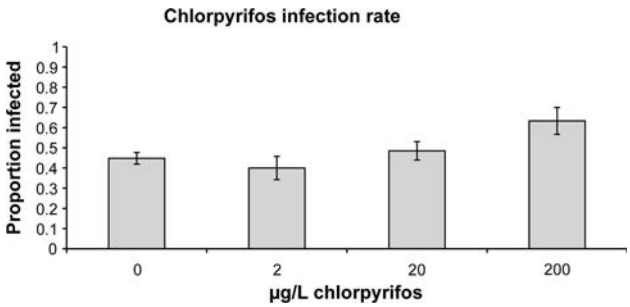

Figure 4. Proportion of larvae infected by virus at each concentration of chlorpyrifos. Atrazine concentrations are pooled for each level of chlorpyrifos, and bars represent ±1 standard error.

($F_{3,6}$ = 10.25, p = 0.01; Figure 4), whereas atrazine had no significant effect ($F_{2,6}$ = 2.69, p = 0.14).

Quantitative PCR estimates of viral loads among individuals show that death corresponds strongly to infection in virus exposed treatments. Dead individuals in the virus treatment exhibited significantly higher viral loads ($F_{1,57}$ = 7.12, p = 0.01) than surviving individuals, with 86% of dead exhibiting greater than 1 × 10^6 viral genome copies. Analysis comparing viral loads did not signify differences with exposure to atrazine ($F_{2,104}$ = 0.24, p = 0.79) or chlorpyrifos ($F_{3,104}$ = 0.34, p = 0.8), or to an interactive effect of both pesticides ($F_{6,104}$ = 0.29, p = 0.94). Examination of infected only animals also did not signify difference in viral loads with exposure to atrazine ($F_{2,47}$ = 0.05, p = 0.95), chlorpyrifos ($F_{3,47}$ = 0.88, p = 0.46), or their interaction ($F_{6,47}$ = 0.85, p = 0.54).

Discussion

Both contaminants and disease are thought to be critical components contributing to amphibian declines (Collins and Storfer, 2003). Several studies have examined the impacts of pesticides on amphibians (for review see Relyea and Hoverman, 2006), but few have investigated their importance in contributing to disease susceptibility and emergence. Our examination of pesticide exposure on a salamander/virus system exhibits key effects otherwise overlooked in single-stressor experiments. Although we did not find any significant interactions between the two pesticides, there were interactions between each pesticide and *Ambystoma tigrinum* virus treatments. Specifically, this study showed three key results concerning the effects of chlorpyrifos: (1) a direct lethal impact on 4-week-old salamander larvae at 200 µg/L; (2) increased susceptibility to viral infection; and (3) an increased additive mortality effect when combined with virus. Atrazine exhibited mixed results by increasing survival slightly in no-virus treatments, and yet slightly decreasing survival in virus exposed treatments. Following, we discuss each of these results in detail.

Impacts of Chlorpyrifos

The widely used insecticide chlorpyrifos has significant impacts on the survival of young tiger salamander larvae, both in the absence and presence of ATV exposure. On its own, chlorpyrifos results in a 20% reduced survival rate at the highest concentration (Figure 1). When this concentration is combined with virus, larval survival decreases >60% compared with the control. We believe this dramatic decrease is primarily the result of increased susceptibility of salamander larvae to viral infection, as suggested by an increased infection rate among exposed larvae (Figure 4), as well as high viral loads among those that died. Although no statistical interactive effect on mortality between chlorpyrifos and viral exposure was detected, >80% of dead individuals exhibited viral loads typical of a diseased individual (>1 × 10^6 viral copies/genome), suggesting that the combination of virus and pesticide exposure interact with one another. One would anticipate decreased viral loads with increasing pesticide concentrations if pesticides were acting separately to kill the salamanders more quickly. Instead, we see an increase in infection rate with increasing concentrations (Figure 4). Further study incorporating increased sample sizes would likely provide increased power to statistically detect an interactive effect. Nonetheless, combined ATV and chlorpyrifos exposure results in decreased survival and increased infection rate, even at the lowest tested concentration (2 µg/L) of chlorpyrifos (Figure 1).

Impacts of Atrazine

Several studies have exhibited impacts of atrazine on amphibians, including alterations of development, behavior, and survival (Hayes et al., 2002; Storrs and Kiesecker, 2004; Rohr et al., 2006). Previous work has shown that atrazine increases disease susceptibility in tiger salamanders (Forson and Storfer, 2006a). Relative to this work, our study was performed on younger larvae (4 vs. 12 weeks), thus we expected to find an even greater susceptibility to atrazine due to both reduced overall size and incompletely developed immune systems (Charlemagne, 1979). Inter-

estingly, we found an interactive effect occurring where survival slightly decreased when atrazine was combined with virus, but actually increased when no virus was present (Figure 2). Admittedly, these slight changes (~10%) on their own may not amount to meaningful impacts to natural population dynamics. Increased mortality in the presence of combined virus and atrazine corroborates early findings on the potential negative impacts on larval susceptibility (Forson and Storfer, 2006a). The increased survival in atrazine treatments has been found in other amphibian studies (Forson and Storfer, 2006b; Storrs and Kiesecker, 2004), although the mechanism for this increased survival is not known.

Atrazine Interaction with Chlorpyrifos

Unlike previous studies (Belden and Lydy, 2000; Wacksman et al., 2006), we did not detect any enhancement of toxicity of chlorpyrifos by atrazine in control or virus-exposed treatments. This corroborates findings on two amphibian species (*Rana clamitans*), which also did not exhibit enhanced toxicity effects of the two pesticides when combined (Wacksman et al., 2006). The atrazine concentrations used in this study were at least five times lower than the concentration used in the study by Wacksman et al., (1,000 μg/L), and therefore enhanced toxicity on *A. tigrinum* might still occur at higher levels of exposure. However, our high level (200 μg/L) represents the ecologically relevant high level found in nature (Hayes et al., 2003).

Despite the lack of a synergistic effect, treatments containing virus and increasing concentrations of the two pesticides exhibited lower survival (Figure 3). A compelling result is the 50% decrease in survival between the virus exposed animals with no pesticides and those with the highest combined concentration treatments (200 μg/L of chlorpyrifos, 20 and 200 μg/L of atrazine). Areas where pulses of multiple pesticides are simultaneously introduced should have significant impacts on viral disease dynamics. In particular, ATV generally follows density-dependent dynamics (Brunner et al., 2004), and an increased proportion of the susceptible population could lead to increased severity of epizootics (Forson and Storfer, 2006a). Future examination of pesticide impacts on disease dynamics should continue to involve multiple pesticides because pesticide use is ubiquitous and pervasive, even in relatively undisturbed habitats.

Conclusions

Changes in parasite densities, as well as host susceptibility and survival, can have dramatic impacts on host-pathogen population dynamics. Slight shifts in these variables can alter the balance often maintained in host-pathogen interactions, driving one to local extinction (McCallum and Dobson, 1995; De Castro and Bolker, 2005). Introduction of pesticides into these systems might provide a key advantage to pathogens by weakening hosts (e.g., immune suppression). Indeed, emerging diseases are increasingly appreciated as a cause of host extinctions (Daszak et al., 2000; De Castro and Bolker, 2005), and the role of environmental cofactors could be important in determining how new epizootics occur.

Typically, disease susceptibility and contaminant impact on amphibians are studied separately. Understanding how these two interact is of crucial interest to deciphering a potential cause of amphibian decline. This study shows that low concentrations of commonly used pesticides can increase tiger salamander susceptibility to an emerging virus. Whereas previous studies have shown nonlethal impacts of combining nutrients and/or pesticides with disease (Johnson et al., 2007; Forson and Storfer, 2006a, b), this is the first to demonstrate lethal impact on amphibians. More field and laboratory-based studies should follow up these results to determine impacts not only in a more natural setting, but also to determine the indirect impacts on the surrounding aquatic communities. It could be that contaminant exposure plays an important, but largely overlooked, role in amphibian disease dynamics.

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