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Reciprocal effects of pesticides and pathogens on amphibian hosts: The importance of exposure order and timing☆

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ABSTRACT

Ecological communities are increasingly exposed to natural and anthropogenic stressors. While the effects of individual stressors have been broadly investigated, there is growing evidence that multiple stressors are frequently encountered underscoring the need to examine interactive effects. Pesticides and infectious diseases are two common stressors that regularly occur together in nature. Given the documented lethal and sublethal effects of each stressor on individuals, there is the potential for interactive effects that alter disease outcomes and pesticide toxicity. Using larval wood frogs (*Lithobates sylvaticus*), we examined the reciprocal interaction between insecticides (carbaryl and thiamethoxam) and the viral pathogen ranavirus by testing whether: (1) prior ranavirus infection influences pesticide toxicity and (2) sublethal pesticide exposure increases susceptibility to and transmission of ranavirus. We found that prior infection with ranavirus increased pesticide toxicity; median lethal concentration (LC50) estimates were reduced by 72 and 55% for carbaryl and thiamethoxam, respectively. Importantly, LC50 estimates were reduced to concentrations found in natural systems. This is the first demonstration that an infection can alter pesticide toxicity. We also found that prior pesticide exposure exacerbated disease-induced mortality by increasing mortality rates, but effects on infection prevalence and transmission of the pathogen were minimal. Collectively, our results underscore the importance of incorporating complexity (i.e. order and timing of exposures) into research examining the interactions between natural and anthropogenic stressors. Given the environmental heterogeneity present in nature, such research will provide a more comprehensive understanding of how stressors affect wildlife.

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1. Introduction

Pesticides are a ubiquitous environmental stressor, with thousands of registered chemicals used worldwide and millions of kilograms of active ingredient applied annually (Grube et al., 2011). These chemicals often enter natural systems, where they influence non-target organisms and disrupt natural processes (Köhler and Triebskorn, 2013; Relyea and Hoverman, 2006). In non-target organisms, pesticides have been linked to endocrine disruption, developmental abnormalities, altered immune function, behavioral changes, and mortality (Brühl et al., 2013; Di Prisco et al., 2013; Egea-Serrano et al., 2012; Gill et al., 2012; Hayes et al., 2010; Mason et al., 2013; McKinlay et al., 2008). Moreover, changes that affect reproduction, survival, and species interactions have been implicated in trophic cascades in terrestrial and aquatic systems (Beketov et al., 2013; Cahill et al., 2008; Chiron et al., 2014; Hallmann et al., 2014; Relyea et al., 2005; Rohr et al., 2008b; Whitehorn et al., 2012). While our understanding of how pesticides influence ecological systems has increased, non-target organisms experience a multitude of stressors, both anthropogenic and natural, which may interact with one another to alter individual physiology, population dynamics, and community structure (Blaustein et al., 2011; Goulson et al., 2015; Koprivnikar, 2010; O'Gorman et al., 2012). A comprehensive understanding of pesticide contamination in ecological systems must therefore incorporate the interactive effects of pesticides and additional stressors.

One stressor in particular that may interact with pesticides is infectious disease. Infectious disease is a fundamental component of ecological communities (Wood and Johnson, 2015). Indeed, wildlife populations encounter a diversity of pathogenic organisms (e.g., viruses, fungi, nematodes) that can influence host morbidity and mortality, population dynamics, and community interactions

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(De Castro and Bolker, 2004; Johnson et al., 2015; Smith et al., 2006). These disease agents often comprise a substantial proportion of biomass in natural systems, perform important functions in food webs, and regulate host population sizes (Kuris et al., 2008; Lafferty et al., 2006; Scott and Dobson, 1989). While infectious diseases are a natural component of communities, there is concern that environmental stressors may exacerbate disease outcomes (Smith et al., 2009, 2006). Anthropogenic stressors such as climate change, habitat alteration, and agrochemical contamination have been implicated in the disruption of infectious disease dynamics by altering the availability of competent hosts, changing optimal environmental conditions for pathogens, and influencing host susceptibility to infection (Bradley and Altizer, 2007; Rohr and Raffel, 2010).

Pesticide contamination has been singled out as a particularly influential stressor because it can influence disease dynamics in a variety of ways (Marcogliese and Pietrock, 2011; Mason et al., 2013). Pesticides can disrupt mechanisms of resistance and tolerance in hosts, often turning relatively benign parasites into pathogenic threats (Marcogliese et al., 2010). Pesticide-induced immunosuppression, namely the reduction of leukocyte counts and down-regulation of immunoregulatory proteins, has been linked to increased disease risk in amphibians, pollinators, and fish (Christin et al., 2003; Di Prisco et al., 2013; Marcogliese et al., 2010). These physiological changes can lead to increased morbidity and mortality in host species (Coors et al., 2008; Rohr et al., 2013). These effects can also cascade through communities by changing host and parasite abundance, as demonstrated with the increase in trematode abundance in wetland communities due to pesticide-mediated increases in intermediate host abundance (Rohr et al., 2008b). While the existing literature provides strong evidence that pesticide contamination can alter disease dynamics in natural systems, there are several gaps in the literature. Previous research has largely focused on susceptibility to infection, yet few studies have addressed the influence of pesticides on parasite transmission between hosts, an important component of disease dynamics (Rohr et al., 2008). Additionally, most studies examine how pesticides alter disease dynamics while few have addressed whether pathogens alter the toxicity of pesticides (Budischak et al., 2009). Given that exposure to pathogens may occur prior to pesticide exposure, infection may damage tissues or modify resource allocation and ultimately alter mechanisms of pesticide tolerance. Infections that damage the liver in particular (e.g., malaria, leishmaniasis) have been shown to reduce xenobiotic metabolizing cytochrome P450s and glutathione s-transferases in rodents, hindering their ability to tolerate chemicals (Ahmad and Srivastava, 2007; Samanta et al., 2003; Tekwanl et al., 1988). Research on coinfecting disease agents has highlighted the importance of priority effects in determining disease outcomes (Hoverman et al., 2013). However, a similar emphasis on order of exposure in pesticide-disease research is needed. In particular, the incorporation of environmental stressors into traditional toxicity tests (e.g., median lethal concentration (LC50) estimates) may provide a more comprehensive understanding of pesticide toxicity in variable environments (Budischak et al., 2009).

Amphibians provide a prime model system for studying pesticide-disease interactions because of the pervasiveness of pesticide contamination in wetland environments and the suite of disease agents implicated in their global population declines (Daszak et al., 2003; Relyea and Hoverman, 2006). Due to the immunosuppressive effects of pesticide exposure, pesticides can increase parasite loads and parasite-induced mortality in larval amphibians (Christin et al., 2003; Koprivnikar, 2010; Rohr et al., 2013, 2008a). Pesticides can also increase exposure to parasites by facilitating the population size of intermediate hosts (e.g., freshwater snails; Rohr et al., 2008b). Consequently, pesticide concentrations in wetlands have been found to be the primary driver of parasite abundance in amphibian populations (Rohr et al., 2008b). Collectively, this research demonstrates that pesticides can alter disease dynamics in amphibians, yet most of this research has focused on trematodes and the fungal pathogen *Batrachochytrium dendrobatidis*. The influence of pesticides on ranavirus, a widespread amphibian disease agent, has been largely understudied.

Ranaviruses are viral pathogens of amphibians that infect the liver, kidney, and spleen and cause edema, lesions, and hemorrhaging, often leading to death (Bollinger et al., 1999; Docherty et al., 2003; Jancovich et al., 1997). Moreover, they have been implicated in worldwide mass mortality events (Ariel et al., 2009; Fox et al., 2006; Green et al., 2002; Une et al., 2009). While pesticides have been implicated as drivers of disease emergence, few studies have experimentally tested the interaction between ranavirus and pesticides. Interestingly, studies that have examined this interaction have found conflicting results. For example, pesticides were shown to increase ranavirus susceptibility in tiger salamanders (*Ambystoma tigrinum*; Forson and Storfer, 2006a; Kerby and Storfer, 2009) but decreased susceptibility in long-toed salamanders (*Ambystoma macrodactylum*; Forson and Storfer, 2006b). Pesticide-induced immunosuppression was argued to be the leading driver of increased ranavirus susceptibility (Forson and Storfer, 2006a), while pesticide-induced immunostimulation and a potential reduction in viral efficacy were proposed as explanations for decreased susceptibility (Forson and Storfer, 2006b). These conflicting results could be due to the experimental designs; individuals were exposed to pesticides and ranavirus simultaneously. With a simultaneous exposure, it becomes difficult to differentiate between the effects of the stressors on the host and the stressors on each other. By controlling the timing and sequence of exposure, we can more directly assess the reciprocal effects of pesticides and ranavirus on amphibians.

The objectives of our study were to determine whether: (1) ranavirus infection affects pesticide toxicity estimates, (2) sublethal pesticide exposure affects ranavirus disease outcomes (e.g., mortality rates, viral load), and (3) sublethal pesticide exposure affects ranavirus transmission. We expected that ranavirus infection would damage host liver and kidney tissues, reducing the ability to metabolize and excrete pesticides, leading to increased pesticide toxicity estimates (lower LC50 values) in infected individuals. If pesticide exposure impairs immune function, we expected an increase in susceptibility to ranavirus indicated by increased mortality rates and viral loads. If increased viral loads resulting from pesticide exposure are observed, we expected this to correlate with an increase in viral shedding rate and transmission to conspecifics.

2. Materials and methods

2.1. Species collection and husbandry

All experiments were carried out using wood frogs, *Lithobates sylvaticus*, collected as 10 partial egg masses from a woodland pond in Nashville, IN on 28 March 2015. Egg masses were reared outdoors in 100-L pools filled with ~70 L of well water and covered with 70% shade cloth. After hatching, tadpoles were fed rabbit chow *ad libitum* until the start of the experiments. Tadpoles were brought inside and acclimated to laboratory conditions (23 °C, 12:12 h day:night photoperiod) for 24 h prior to the start of each experiment. Unless noted otherwise, water changes were conducted every 4 d and tadpoles were fed Tetramin *ad libitum* every 2 d during all experiments.

Ranavirus was isolated from an infected larval green frog, *Lithobates clamitans*, collected from the Purdue Wildlife Area (PWA) in

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West Lafayette, IN. The virus was cultured using a protocol adapted from Hoverman et al. (2010) wherein virus was passaged through fathead minnow cells incubated at 28° C without CO_2 and fed with Eagle's minimum essential medium (MEM) with Hank's salts and 5% fetal bovine serum. The virus was on the second passage since original isolation and was stored at −80 °C until used in the experiments.

2.2. Pesticide application

We selected two insecticides with different modes of action for the study: (1) the carbamate carbaryl, an acetylcholinesterase inhibitor and (2) the neonicotinoid thiamethoxam, a nicotinic acetylcholine receptor agonist. Both insecticides are widely used, with approximately 100,000 to 500,000 kg applied annually in the contiguous U.S. (Baker and Stone, 2015). Because carbaryl is capable of targeting both vertebrate and invertebrate nervous systems, it has been widely studied for its non-target effects on aquatic systems (Story and Cox, 2001). Thiamethoxam represents a newer class of insecticides lauded for its invertebrate specificity (Maienfisch et al., 2001). However, few studies have examined its effects on aquatic systems (Morrissey et al., 2015).

For each experiment, we used commercial grade carbaryl (22.5% Sevin) and thiamethoxam (21.6% Optigard Flex). Lethal concentrations of each pesticide were determined using pilot studies prior to the start of the experiments. We created working solutions by adding 1 mL of pesticide to 9 mL of filtered, UV-irradiated water to achieve 23,600 mg L^{-1} of carbaryl and 24,400 mg L^{-1} of thiamethoxam; experimental concentrations were made by adding working solutions to filtered, UV-irradiated well water. Nominal pesticide concentrations were verified at the Bindley Bioscience Center Metabolite Profiling Facility at Purdue University (Table 1).

2.3. Experiment 1 — effects of ranavirus exposure on LC50 values

We performed LC50 tests to determine the effects of ranavirus exposure on pesticide toxicity estimates. Our experiment was a randomized factorial design consisting of seven pesticide treatments and two virus treatments. The pesticide treatments consisted of a control (no pesticide) and three concentrations (0.3, 3, and 30 mg L^{-1}) of each pesticide. These concentrations allowed us to measure both the expected LC50 values for healthy individuals and the theoretically reduced values for infected individuals, while providing the minimum number of concentrations needed to produce statistically sound results. The ranavirus treatments consisted of a no-virus control and exposure to ranavirus at a concentration of 10^3 PFU mL^{-1}. Experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated aged well water. We randomly assigned 10 tadpoles at Gosner stage 28 (Gosner, 1960) to each unit. We replicated the 14 treatments four times for a total of 56 experimental units.

We began the experiment by adding 1.43 mL of the virus (original titer 7×10^5 PFU mL^{-1}) to each virus treatment to achieve a final concentration of 10^3 PFU mL^{-1}. Previous studies have demonstrated that this dosage is sufficient for initiating infection in wood frogs and other ranids (Hoverman et al., 2011, 2010). For instance, 95% infection prevalence was documented using identical exposure conditions (Hoverman et al., 2011). We added 1.43 mL of MEM to the experimental units not assigned to the virus treatment to serve as a control. After 24 h, tadpoles were moved to new containers containing fresh water for 3 d before conducting the LC50 test. We chose to begin the LC50 test on day 4 of ranavirus exposure because we wanted to examine pesticide toxicity after virus infection, but before individuals experienced disease-induced mortality. Previous work has demonstrated that mortality due to ranavirus increases sharply on day 7 following exposure (Hoverman et al., 2011). Given the 48 h window of the LC50 test, the 4 d ranavirus exposure period allowed us to detect treatment differences before the day 7 mortality spike would occur.

The LC50 tests were initiated on day 4 by randomly assigning experimental units from each virus treatment to the pesticide treatments. We applied the pesticide concentrations to the experimental units and tadpoles were subsequently monitored for mortality every 8 h for 48 h. Dead individuals were removed and preserved in 70% ethanol. At the end of the experiment, all individuals were euthanized using a 2 g L^{-1} solution of MS-222 and preserved in 70% ethanol. A randomly selected subset of 4 tadpoles from each treatment was tested to ensure infection in ranavirus-exposed tadpoles and no infection in control tadpoles.

2.4. Experiment 2 — effects of pesticides on ranavirus susceptibility

To determine the effect of pesticide exposure on susceptibility to ranavirus, we conducted a randomized factorial experiment consisting of three pesticide treatments and three ranavirus treatments. The pesticide treatments consisted of a control (no pesticide) and exposure to carbaryl (1 mg L^{-1}) or thiamethoxam (1 mg L^{-1}). These concentrations were sublethal to tadpoles in our pilot studies and are both representative of concentrations measured in natural surface waters (Main et al., 2014; Norris et al., 1983). Ranavirus treatments consisted of a no-virus control, immediate exposure to ranavirus at a concentration of 10^3 PFU mL^{-1} following pesticide exposure, and ranavirus exposure (10^3 PFU mL^{-1}) 14 days following pesticide exposure. The two exposures were chosen to determine if ranavirus susceptibility changes with time since pesticide exposure, with 14 days chosen to avoid allowing tadpoles to metamorphose. The experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated aged well water. We randomly assigned 10 tadpoles at Gosner stage 29 (Gosner, 1960) to each unit. We replicated each treatment four times for a total of 36 experimental units.

We exposed tadpoles to their respective pesticide treatments for 7 d, which has been shown to be sufficient in altering susceptibility to infection (Rohr et al., 2008a), and pesticide solutions were

Table 1
Nominal and actual concentrations of carbaryl and thiamethoxam.

Insecticide (common name; % active ingredient)	Nominal Concentration	Actual Concentration
Carbaryl (Sevin; 22.5%)	0.3 mg L^{-1}	0.2 mg L^{-1}
	1.0 mg L^{-1}	0.8 mg L^{-1}
	3.0 mg L^{-1}	1.7 mg L^{-1}
	30.0 mg L^{-1}	14.3 mg L^{-1}
Thiamethoxam (Optigard Flex; 21.6%)	0.3 mg L^{-1}	0.2 mg L^{-1}
	1.0 mg L^{-1}	0.7 mg L^{-1}
	3.0 mg L^{-1}	2.3 mg L^{-1}
	30.0 mg L^{-1}	25.2 mg L^{-1}

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renewed with each water change. Given the estimated half life of each pesticide, concentrations were expected to remain fairly stable between water changes (carbaryl, 10 d at pH = 7; thiamethoxam, 200 d at pH = 7; Maienfisch et al., 2001). After 7 d, tadpoles were moved to fresh water and exposed to their respective virus treatments. Tadpoles in the immediate virus exposure treatment were exposed to virus immediately after pesticide exposure on day 8. We added 1.43 mL of the virus (original titer 7×10^5 PFU mL^{-1}) to achieve a final concentration of 10^3 PFU mL^{-1}. Tadpoles in the delayed virus exposure treatment remained in fresh water for 2 wk before being exposed to virus on day 22 (10^3 PFU mL^{-1}). We added 1.43 mL of MEM to the experimental units not assigned to the virus treatment to serve as a control. After 24 h of virus exposure, the tadpoles were moved to fresh water for the remainder of the experiment. Tadpoles in the virus treatments were monitored for mortality every 12 h until 100% mortality was observed. Dead individuals were immediately removed and preserved in 70% ethanol for ranavirus testing. At the end of the experiment, surviving individuals were euthanized with MS-222 and preserved in 70% ethanol.

Each individual was weighed, measured for snout-vent length (SVL) and total length, and staged. Then, the individual was necropsied and sections of the liver and kidney were pooled into one 1.5 mL microcentrifuge tube for ranavirus testing. From each sample, we extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen) and stored at −80°C until qPCR analysis. To prevent cross contamination during necropsies, we scrubbed and soaked all tools and surfaces in 10% bleach for 10 min and changed gloves between samples.

2.5. Experiment 3 — effects of pesticides on ranavirus transmission

To determine the effect of pesticide exposure on the transmission of ranavirus, we conducted an experiment analyzing two components of ranavirus transmission from a focal host to a naïve host: (1) viral shedding rate of the focal host and (2) infection in naïve hosts. The experiment was a completely randomized 3 × 2 factorial design manipulating pesticide and ranavirus exposure on the focal tadpoles. The pesticide treatments consisted of a control (no pesticide) and sublethal exposure to carbaryl or thiamethoxam (1.0 mg L^{-1}). The ranavirus treatments consisted of a no-virus control and exposure to ranavirus at a concentration of 10^3 PFU mL^{-1}. We replicated each treatment 10 times for a total of 60 experimental units. The experimental units were 2-L plastic tubs filled with 1 L of filtered, UV-irradiated well water aged for 24 h prior to use. We randomly assigned one focal tadpole to each experimental unit.

We exposed focal tadpoles to their respective pesticide treatments for 7 d followed by virus exposure for 24 h. After exposure to ranavirus for 24 h, tadpoles were rinsed with fresh water and moved to new containers with fresh water to ensure no virions from the initial exposure remained in the tubs. Every 24 h for 3 d, 40 mL water samples were taken from each experimental unit and frozen at −80°C to test for ranavirus. We stirred the water in each unit before sampling to ensure homogeneity, and changed water after each sampling. After 3 d, focal tadpoles were euthanized using MS-222 and stored in 70% ethanol for ranavirus testing. Water from the experimental units was kept unchanged for the next portion of the experiment. To each experimental unit, we added 5 naïve tadpoles, which had never been exposed to pesticides or virus. Naïve tadpoles were maintained in the contaminated water for 3 d before being euthanized in MS-222 and stored in 70% ethanol for ranavirus testing. Tadpoles were processed as described above.

To extract ranavirus from the water samples, we used a protocol adapted from Kirshtein et al. (2007). In brief, the thawed 40 mL water samples were filtered through 0.2 μm PVDF syringe filters. The filters were incubated using DNA extraction reagents (Qiagen). Extracted DNA was transferred to 1.5 mL microcentrifuge tubes and frozen at −80° C until qPCR analysis. All tools and surfaces were soaked in 10% bleach, and gloves and syringes were changed between samples.

2.6. Ranavirus testing

We used quantitative polymerase chain reaction (qPCR) to determine the viral load of each sample using the methods of Forson and Storfer (2006a,b). The PCR reaction mixture included 6.25 μL of TaqMan® Universal PCR Master Mix (Applied Biosystems), 2.75 μL of DNA grade water, 1.0 μL of a mixture of each primer at 10 pmol μL^{-1} (rtMCP-F [5′-ACA CCA CCG CCC AAA AGT AC-3'] and rtMCP-R [5′-CCG TTC ATG ATG CGG ATA ATG-3']) and a fluorescent probe rtMCP-probe (5′- CCT CAT CGT TCT GGC CAT CAA CCA-3′). Each well included 2.5 μL of its respective template DNA or DNA grade water for a final volume of 12.25 μL. We ran qPCR reactions using a Bio-Rad real-time PCR system. Each qPCR run included a standard curve and a negative control. The DNA standard was a synthetic double-stranded 250bp fragment of the highly conserved *Ranavirus* major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies). A standard curve was created using a log-based dilution series of 4.014×10^9 viral copies μL^{-1} to 4.014×10^6 viral copies μL^{-1}. All samples, including standard curves, negative controls, and unknowns, were run in duplicate. For each sample, the concentration of genomic DNA (ng of DNA μL^{-1}) was measured using a NanoDrop 2000c (Thermo Scientific). Using these measurements, we calculated viral load as viral copies ng^{-1} of DNA.

2.7. Statistical analyses

To compare LC50 values in experiment 1, we followed the methods of Budischak et al. (2009). Experimental units from each virus treatment were randomly assigned to cohorts such that each cohort contained the full range of pesticide concentrations (0, 0.3, 3, and 30 mg L^{-1}). We calculated LC50 values for each cohort individually using probit analysis, which produced four replicate LC50 values for each virus treatment. We used individual one-way analyses of variance (ANOVAs) to compare LC50 values between virus and no-virus treatments for each pesticide separately. LC50 estimates were adjusted according to the actual verified pesticide concentrations. For all analyses in experiment 2, we used general linear mixed models with experimental unit as a random factor. Of our size measurements, SVL was found to have the strongest positive correlation with time to death and viral load ($p < 0.02$) and was therefore included as a covariate in our analyses for experiment 2. Separately for each virus exposure regime, we compared time to death among pesticide treatments. Additionally, we examined the relationship between time to death and viral load in our treatments using general linear mixed models with experimental unit as a random factor. This was done to determine whether we could assess the effects of pesticide exposure on tolerance (Read et al., 2008). Overall, there was no relationship between time to death and viral load in our treatments ($P > 0.08$). The one exception was for individuals exposed to thiamethoxam immediately before ranavirus exposure. In this treatment, there was a positive relationship between viral load and time to death ($F_{1,35} = 7.76$, $p = 0.01$). We expect that this is a result of *in vivo* viral replication over time, where individuals that survived longer had higher viral loads. Given the general lack of a relationship between viral load and time to death, we did not explore additional analyses of tolerance but focus instead on the effects of pesticide exposure

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on time to death. For individuals across both virus exposure regimes, we determined if there was an interactive effect of pesticide and the timing of virus exposure on time to death. For this test, time to death was inverse transformed to meet the assumption of homoscedasticity. We also compared viral load among pesticide treatments. For experiment 3, we assessed the effects of pesticide treatment on the mean viral load of focal and naïve tadpoles with one-way ANOVAs. The no-virus treatment was excluded from the analysis because no individuals were infected. Based on Pearson's correlations, none of our size variables were correlated with viral load ($p > 0.06$) and were therefore excluded from the analyses for this experiment. In analyzing viral loads of the naïve tadpoles, we calculated the mean viral load for all tadpoles housed within each experimental unit. Because viral concentrations in the water samples were too low to be detected, no statistical analyses were conducted. All analyses were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA) at $\alpha = 0.05$.

3. Results

3.1. Experiment 1 – effects of ranavirus exposure on LC50 values

Virus exposure significantly increased the toxicity of carbaryl ($F_{1,6} = 23.06$, $p = 0.003$) and thiamethoxam ($F_{1,6} = 11.65$, $p = 0.01$; Fig. 1, Fig. A1). LC50 estimates were 72% and 55% lower in the virus treatment for carbaryl and thiamethoxam, respectively, compared to the no-virus treatments. We observed 100% infection in the ranavirus treatment and 0% infection in the no-virus control based on a randomly selected subset of tadpoles from each treatment. Within this subsample, there was no effect of pesticide treatment on viral load ($F_{2,30} = 1.27$, $p = 0.30$).

3.2. Experiment 2 – effects of pesticides on ranavirus susceptibility

Time to death decreased (i.e. tadpoles died faster) when tadpoles were exposed to pesticides prior to ranavirus infection ($F_{2,9} = 3.75$, $p = 0.07$; Fig. 2a). However, the effect was dependent on the pesticide. Based on post-hoc comparisons, carbaryl significantly decreased time to death compared to control ($p = 0.02$) but thiamethoxam did not ($p = 0.16$). When ranavirus exposure was delayed 2 wk following pesticide exposure, there was no effect of the pesticide treatments on time to death ($F_{2,8} = 2.97$, $p = 0.11$; Fig. 2b). Moreover, time to death was significantly higher in the delayed exposure ($F_{1,53} = 105.94$, $p < 0.001$), and there was an interactive effect of pesticide and the timing of virus exposure on time to death ($F_{2,24} = 6.26$, $p = 0.01$). However, in both the immediate and delayed exposures, pesticide exposure did not influence infection prevalence (100% of tadpoles were infected in the ranavirus treatment) or viral load at time of death (Immediate, $F_{2,9} = 0.15$, $p = 0.87$; Delayed, $F_{2,9} = 3.24$, $p = 0.09$; Fig. 3). Furthermore, there was no difference in viral load between the immediate and delayed exposure regimes ($F_{1,39} = 0.75$, $p = 0.39$).

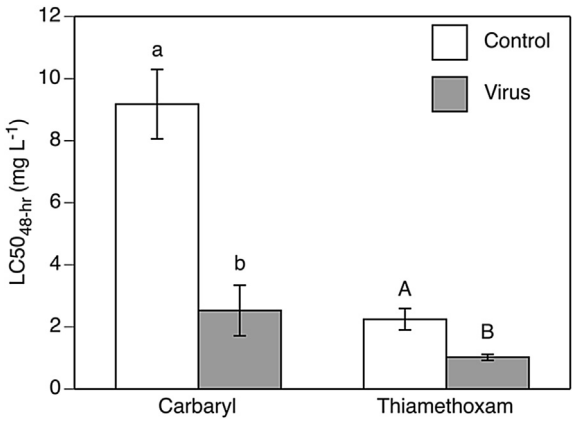

Fig. 1. $LC50_{48\text{-hr}}$ values for carbaryl and thiamethoxam for ranavirus-exposed and unexposed larval wood frogs. Treatments sharing uppercase or lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

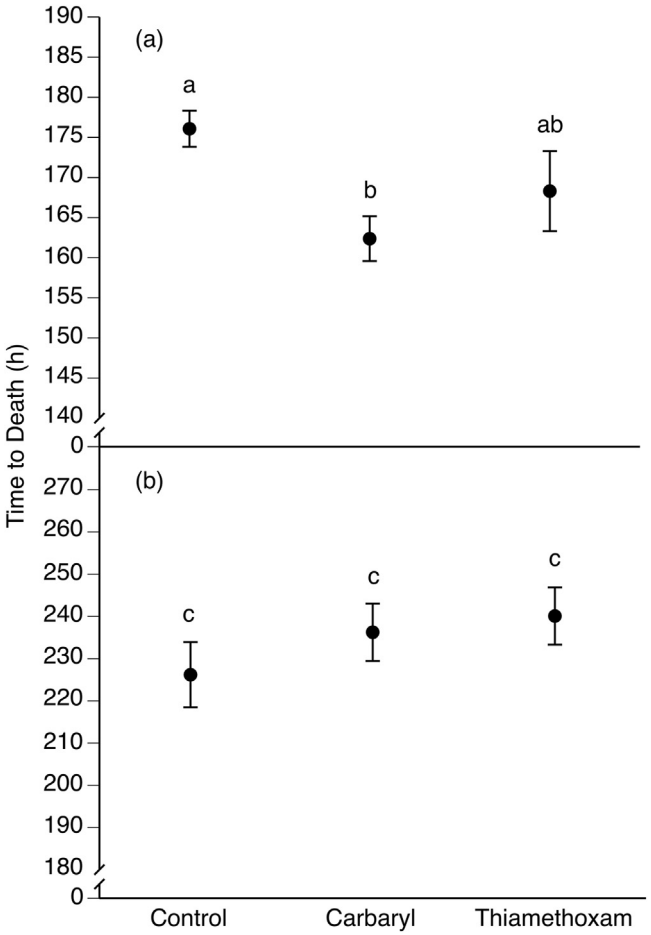

Fig. 2. Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. (a) Individuals were exposed to ranavirus immediately after pesticide exposure. (b) Individuals were exposed to ranavirus 2 wk after pesticide exposure. Treatments sharing lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

3.3. Experiment 3 – effects of pesticides on ranavirus transmission

Sublethal pesticide exposure had no effect on the viral load of the focal tadpoles ($F_{2,27} = 4.01$, $p = 0.14$; Fig. 4). All focal hosts exposed to ranavirus were infected with an average viral load of 75,892 viral copies ng DNA^{-1}. While we were unable to detect shed virions in the water of the focal tadpoles, there was evidence of transmission to the naïve tadpoles because 100% of naïve tadpoles were infected with ranavirus. Additionally, the viral load of naïve tadpoles differed among pesticide treatments ($F_{2,27} = 5.44$ $p = 0.01$; Fig. 4). Compared to the control, viral load was lower in the carbaryl treatment ($p = 0.006$). There was no difference between the control and thiamethoxam treatments ($p = 0.79$). Finally, mean viral load was 65% lower in naïve tadpoles compared to focal tadpoles.

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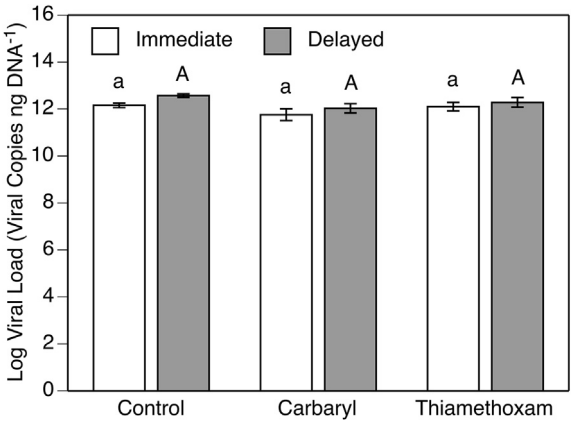

Fig. 3. Viral load (viral copies ng DNA^{-1}) at time of death for ranavirus-exposed larval wood frogs that were previously exposed to no pesticides (control), carbaryl (1 mg L^{-1}) or thiamethoxam (1 mg L^{-1}). Individuals were either exposed to ranavirus immediately ("Immediate") after pesticide exposure or 2 wk after pesticide exposure ("Delayed"). Treatments sharing uppercase and lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

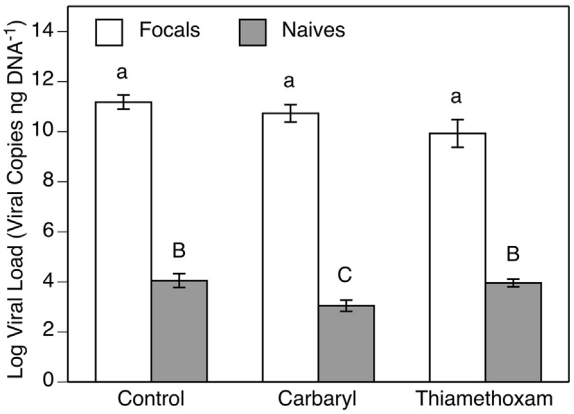

Fig. 4. Viral load (viral copies ng DNA^{-1}) at time of death for ranavirus-infected focal and naïve larval wood frogs. Focal larvae were previously exposed to one of three insecticide treatments (a control, carbaryl at 1 mg L^{-1}, or thiamethoxam at 1 mg L^{-1}) before virus addition. Naïve larvae were not previously exposed to insecticides or ranavirus before addition to containers with water from focals. Treatments sharing uppercase or lowercase letters are not statistically different ($p > 0.05$). Data are means ± 1 SE.

4. Discussion

There is a growing interest in addressing the interactive effects of pesticide exposure and disease on hosts. While there is evidence for altered disease dynamics as a result of pesticide exposure across host taxa, considerable research is needed for many understudied disease systems (Coors et al., 2008; Di Prisco et al., 2013; Marcogliese et al., 2010; Rohr et al., 2013). Moreover, research that addresses the effects of prior infection on estimates of pesticide toxicity is needed. We examined these interactions in the amphibian-ranavirus system, focusing both on the effects of pesticides on ranavirus dynamics and the effects of ranavirus infection on pesticide toxicity. We found that prior ranavirus infection can increase pesticide toxicity, and that pesticide exposure can alter disease outcomes.

We found that prior ranavirus infection increased the toxicity of the insecticides carbaryl and thiamethoxam to larval wood frogs by 72% and 55%, respectively. Notably, infection shifted LC50 values to concentrations measured in surface waters for thiamethoxam (~2.0 mg L^{-1}; J. Hoverman, M. Sepúlveda, and C. Krupke, unpublished data) and carbaryl (4.8 mg L^{-1}; Norris et al., 1983). Given the widespread prevalence of ranavirus infection and the ubiquity of pesticide contamination, this interaction could have considerable impacts on amphibian populations (Ariel et al., 2009; Fox et al., 2006; Green et al., 2002; Une et al., 2009). Because many pesticides have immunosuppressive effects on non-target organisms, research on pesticide-disease interactions has primarily focused on pesticide-mediated effects on disease outcomes (Christin et al., 2003; Di Prisco et al., 2013; Mason et al., 2013). While these effects are important, they assume that hosts are exposed to pesticides prior to disease agents. However, wild populations are likely to experience temporally varied exposure to pesticides and disease agents. Our results underscore the importance of considering scenarios in which pesticide exposure occurs following infection. Additionally, our results highlight the value in incorporating natural stressors into measurements of toxicity. Traditional toxicity tests, such as LC50 determinations, generally exclude the effects of natural stressors. However, by considering these effects, we can gain a better understanding of contaminant toxicity in natural environments. Similar effects on pesticide-induced mortality have been found for other stressors, such as predator cues (Relyea and Mills, 2001), but the effect of disease has rarely been addressed (Budischak et al., 2009). Given the ubiquity of parasites in natural systems, there is a need for further investigation involving other species and disease systems.

We also found that prior exposure to pesticides can influence disease outcomes in wood frogs. However, these effects were dependent on the pesticide and timing of ranavirus exposure following pesticide exposure. Time to death for tadpoles exposed to carbaryl was 8% shorter compared to control tadpoles. However, we did not observe this effect with thiamethoxam. Moreover, when the ranavirus exposure occurred two weeks post pesticide exposure, neither pesticide influenced time to death. These results suggest that pesticide exposure can influence disease-induced mortality, but the effects can be eliminated if individuals are given the opportunity to metabolize pesticides. Importantly, these results were not influenced by differences in susceptibility to infection; all individuals exposed to ranavirus become infected. Conversely, Forson and Storfer (2006a, 2006b) found that simultaneous exposure to the herbicide atrazine altered susceptibility to ranavirus infection in ambystomatid salamanders. Additionally, Rohr et al. (2013) determined that early-life exposure to atrazine increased *Bd*-induced mortality in later developmental stages of Cuban treefrogs, indicating that pesticide metabolism did not ameliorate mortality effects. However, differences in species, disease agents, pesticide modes of action, and order of exposure may all contribute to variation in susceptibility and mortality effects. In comparing viral load among pesticide treatments, we found no differences in both the immediate and delayed exposure regimes. Given that all measurements were taken at time to death, this indicates that individuals may experience mortality at similar viral loads. Additionally, wood frogs are highly susceptible to ranavirus infection with case mortality rates >95% (Hoverman et al., 2011), which may explain why there were no detectable differences in viral load. Because there is considerable variability in ranavirus dynamics among species (Hoverman et al., 2011), there is a need for research on other amphibian species to assess generality. For example, Forson and Storfer (2006a) also found that pesticide exposure did not affect viral load in ranavirus-infected tiger salamanders, suggesting that this may be a general trend for the amphibian-ranavirus system. Conversely, in other systems, pesticides have been shown to increase viral load, as seen with honey bees infected with deformed wing virus (Di Prisco et al., 2013). Infecting individuals with lower viral concentrations may also aid in detecting

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subtle changes in viral load by preventing individuals from reaching the high viral load threshold where they appear to experience mortality. Future studies that generate variability in mortality and viral load will be necessary to determine how pesticide exposure affects the relationship between host fitness and parasite burden (i.e. tolerance of infection; Read et al., 2008). Collectively, our results suggest that pesticide exposure can increase disease-induced mortality rates, but this effect may be ameliorated if there is sufficient time to metabolize pesticides before pathogen exposure.

In addition to susceptibility, we examined the effects of pesticide exposure on ranavirus transmission. We found no effect of pesticide exposure on the viral load in focal hosts, suggesting that any differences in transmission were not due to pesticide-mediated effects on ranavirus infection. We did not recover ranavirus from the water samples and could not determine if ranavirus shedding rates differed among pesticide treatments. However, it was clear that transmission occurred because all naïve hosts were infected following exposure to water from the focal hosts. There were no differences in infection success among the naïve hosts, but viral loads were lower for naïve hosts in the carbaryl treatment. Therefore, pesticide exposure may affect transmission dynamics, either by affecting shedding rate or by affecting the virulence of shed particles. Viral shedding rates may be fairly low because we were unable to detect virus concentrations in the water. Additionally, viral loads for the naïve hosts were considerably lower than for the directly infected focal hosts. To our knowledge, there are no previous studies investigating ranavirus shedding rates. Therefore, considerable work is needed to understand this route of exposure and the influence of pesticide contamination.

5. Conclusions

Across taxa, species experience a variety of natural and anthropogenic stressors that may co-occur and interact, often with variable outcomes. For example, predator stress can magnify the effects of pesticides, ameliorate these effects, or influence how future generations respond to pesticide exposure (Gergs et al., 2013; Relyea, 2012; Trekels et al., 2013). Given the highly context-dependent nature of multiple stressor interactions, there is a need for research that addresses the details of these interactions to fully understand how they might influence species. We found that pesticide exposure and ranavirus infection have interactive effects on an amphibian host, and importantly, these effects are sensitive to the order and timing of exposure, providing further evidence that stressors can interact in context-dependent ways. When pesticide exposure preceded ranavirus infection, disease-induced mortality rates increased. Moreover, when we reversed the order of exposure, prior ranavirus infection increased the toxicity of pesticides and lowered LC50 values to environmentally relevant concentrations. In disease systems, we see similar priority effects when host organisms are coinfected with multiple pathogens in different orders (Hoverman et al., 2013), but rarely is a connection drawn to pesticide-disease interactions. These results emphasize the value of addressing these priority effects in studies of pesticides and disease dynamics by utilizing study designs that manipulate the order and timing of exposure. Additionally, they highlight the importance of incorporating natural stressors into traditional toxicity tests, which generally do not account for environmentally relevant scenarios. Given the multitude of natural and anthropogenic stressors that commonly co-occur and the context-dependency of their interactions, it is imperative that we form a comprehensive understanding of how stressors interact in varied systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.11.086.

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Appendix A

Appendix Figure Legends

Figure A1. Mean percent survival of larval wood frogs across pesticide and ranavirus treatments. Individuals were either exposed or not exposed to ranavirus followed by exposure to pesticide at one of four concentrations **(**0.0, 0.3, 3.0, 30.0 mg L^{-1}). Carbaryl and thiamethoxam are represented in separate columns. Pesticide concentrations are represented in separate rows. A common pesticide control (0.0 mg L^{-1}) was used and is therefore presented twice in this figure, once for each pesticide. Data are means ± 1 SE.

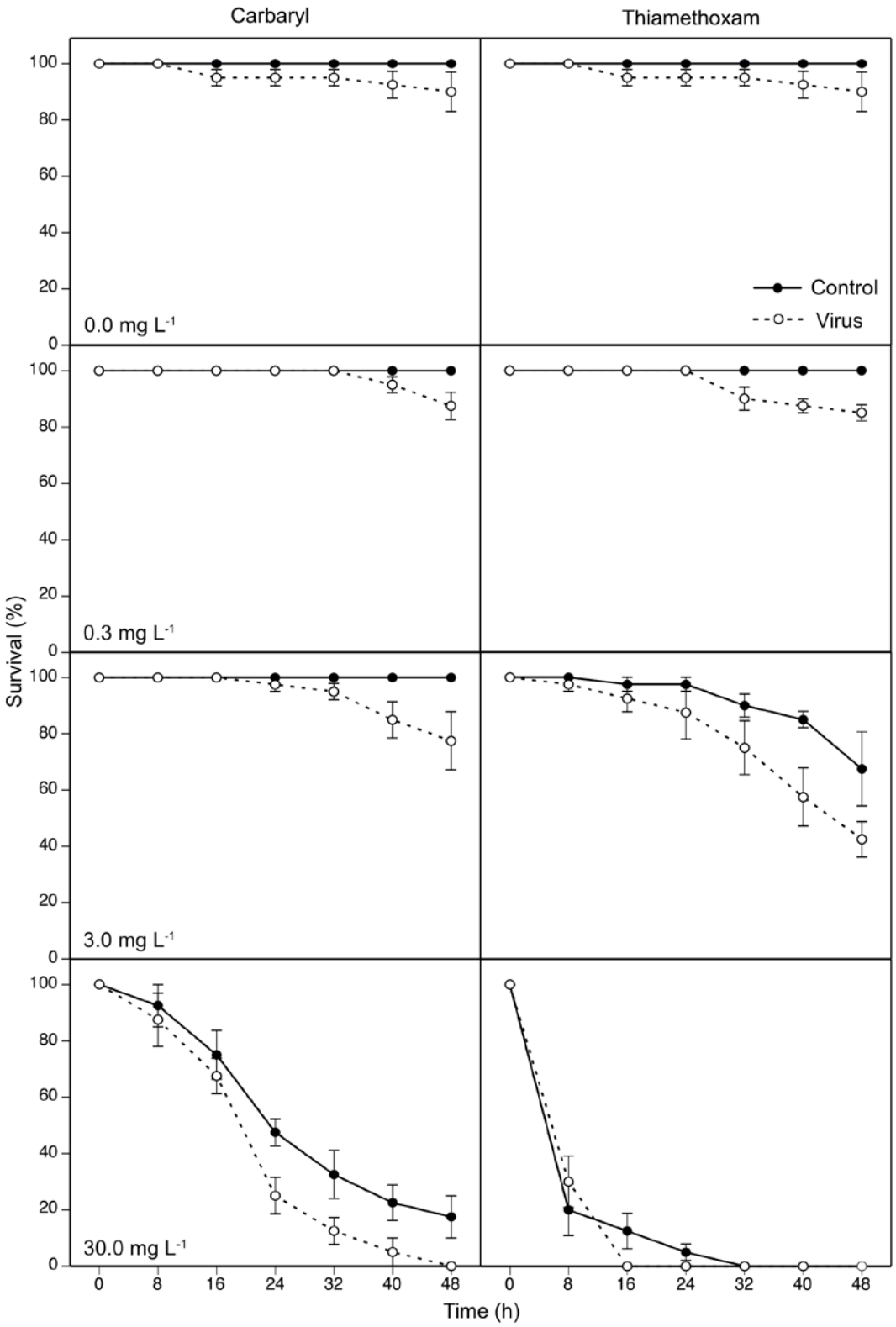
Carbaryl
Thiamethoxam
Control
Virus
0.0 mg L-1
0.3 mg L-1
3.0 mg L-1
30.0 mg L-1
Survival (%)
Time (h)
0
20
40
60
80
100
0
8
16
24
32
40
48

Figure A1