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The interactive effects of pesticide exposure and infectious disease on amphibian hosts

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For the degree of Master of Science

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Approved by Major Professor(s): Jason T. Hoverman

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Head of the Departmental Graduate Program Date

THE INTERACTIVE EFFECTS OF PESTICIDE EXPOSURE AND INFECTIOUS
DISEASE ON AMPHIBIAN HOSTS

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of

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Katherine M. Pochini

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TABLE OF CONTENTS

	Page
LIST OF TABLES	iv
LIST OF FIGURES	v
ABSTRACT	vii
CHAPTER 1. PRIOR RANAVIRUS INFECTION INCREASES PESTICIDE TOXICITY IN AMPHIBIANS: INTERACTIVE EFFECTS OF A WIDESPREAD DISEASE AND PESTICIDES	1
1.1 Abstract	1
1.2 Introduction	2
1.3 Materials and Methods	5
1.4 Results	11
1.5 Discussion	12
1.6 Literature Cited	16
CHAPTER 2. PESTICIDES INFLUENCE THE TOLERANCE OF AMPHIBIAN HOSTS TO TREMATODE INFECTIONS.....	28
2.1 Abstract	28
2.2 Introduction	30
2.3 Materials and Methods	33
2.4 Results	40
2.5 Discussion	41
2.6 Literature Cited	45
CHAPTER 3. CONCLUSIONS	58
3.1 Conclusions and Future Directions	58
3.2 Literature Cited	60

LIST OF TABLES

Table	Page
Table 1.1 Nominal and actual concentrations of carbaryl and thiamethoxam.....	22

LIST OF FIGURES

Figure	Page
Figure 1.1 LC ₅₀ _{48-hr} values for carbaryl and thiamethoxam for ranavirus-exposed and unexposed larval wood frogs. Data are means \pm 1 SE.....	23
Figure 1.2 Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. Individuals were exposed to ranavirus immediately after pesticide exposure. Data are means \pm 1 SE.	24
Figure 1.3 Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. Individuals were exposed to ranavirus 2 wk after pesticide exposure. Data are means \pm 1 SE.	25
Figure 1.4 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”). Data are means \pm 1 SE.	26
Figure 1.5 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. Data are means \pm 1 SE.	27
Figure 2.1 Activity (percent of time spent active) of larval northern leopard frogs across pesticide and parasite treatments. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed). Data are means \pm 1 SE.....	51

Figure	Page
Figure 2.2 Relationship between parasite encystment (percent of the initial exposure amount) and activity (percent of time spent active) of larval northern leopard frogs across pesticide treatments. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed).	52
Figure 2.3 Proportion of parasites encysting in focal larval northern leopard frogs across pesticide treatment. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed). Data are means \pm 1 SE.	53
Figure 2.4 Proportion of northern leopard frogs surviving to metamorphosis within experimental tanks across pesticide and parasite treatments. Data are means \pm 1 SE.....	54
Figure 2.5 Relationship between parasite load and day of metamorphosis for larval northern leopard frogs across pesticide treatments. Day of metamorphosis was log transformed + 1 to meet the assumption of linearity.	55
Figure 2.6 Relationship between parasite load and mass at metamorphosis for larval northern leopard frogs across pesticide treatments.	56
Figure 2.7 Proportion of parasites encysting in larval northern leopard frogs across time points and pesticide treatment. (a) Larvae were exposed to pesticide prior to being infected with <i>Echinoparyphium</i> cercariae. (b) <i>Echinoparyphium</i> cercariae were exposed to pesticide prior to infecting larvae. Data are means \pm 1 SE.	57

ABSTRACT

Pochini, Katherine M. M.S., Purdue University, August 2016. The Interactive Effects of Pesticide Exposure and Infectious Disease on Amphibian Hosts. Major Professor: Jason Hoverman.

Natural systems are home to a multitude of natural and anthropogenic stressors, which draw an array of effects on ecological communities. While these effects have been investigated individually, it is important, given the routine co-occurrence of these stressors, to understand their interactive effects. Pesticide exposure and infectious disease are two common, co-occurring stressors that each have documented detrimental effects on species and, as evidence suggests, may have interactive effects. Moreover, existing research suggests that these interactive effects are highly context dependent, eliciting different results based on species, disease agent, toxin, and environment. Given the variability with which species may experience multiple stressors, it is imperative that we form a detailed understanding of these interactions in diverse systems. Amphibians are an ideal study system due to the pervasiveness of pesticide contamination in wetland habitats and the various disease agents contributing to their global population declines. Here I sought to contribute a more comprehensive understanding of pesticide-disease interactions in amphibians by (1) examining this interaction using an understudied disease agent, ranavirus, and (2) exploring how pesticides affect the mechanisms by which hosts and parasites increase their fitness during their relationship. In a series of experiments using larval wood frogs (*Lithobates sylvaticus*) and two insecticides (carbaryl and thiamethoxam), I found that prior ranavirus infection increased the toxicity of both pesticides, reducing median lethal concentrations (LC50 estimates) by 72 and 55% for carbaryl and thiamethoxam, respectively. Importantly, these reductions matched concentrations found in natural surface waters. Moreover, when pesticide exposure

preceded ranavirus infection, I found that carbaryl exacerbated disease-induced mortality. However, these effects were ameliorated if individuals were given the opportunity to metabolize the pesticide. There was minimal effect of pesticides on susceptibility to or transmission of ranavirus. These results highlight the context-dependency of pesticide-disease interactions and emphasize the importance of examining these interactions in detail. To further this idea, I conducted several experiments using larval northern leopard frogs (*Lithobates pipiens*), the trematode *Echinoparyphium spp.*, and carbaryl to examine how pesticide exposure affects both a host's ability to increase its fitness when challenged with a parasite (i.e. resistance and tolerance) and a parasite's ability to successfully infect a host. I found that pesticide exposure of hosts did not affect the resistance mechanisms of parasite avoidance or clearance, and that exposure of parasites did not affect their ability to infect hosts. However, pesticide exposure influenced infection tolerance by decreasing time to metamorphosis in more highly infected individuals by a factor of 31%. Collectively, these results underscore that pesticide-disease interactions are context-dependent and have variable outcomes on hosts and parasites. Importantly, they affirm that individual examinations of stressors, particularly of pesticide toxicity, are not sufficient predictors of the effects of stressors in complex systems. It is essential in a progressively human-influenced environment that research addresses the various ways that stressors interact and the consequences of these interactions for populations and communities.

CHAPTER 1. PRIOR RANAVIRUS INFECTION INCREASES PESTICIDE TOXICITY IN AMPHIBIANS: INTERACTIVE EFFECTS OF A WIDESPREAD DISEASE AND PESTICIDES

1.1 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 both alter disease outcomes and pesticide toxicity. Using larval wood frogs (*Lithobates sylvaticus*), I examined the interaction between insecticides (carbaryl and thiamethoxam) and the viral pathogen ranavirus. I tested whether prior ranavirus infection influences susceptibility to pesticides. Additionally, I tested whether sublethal pesticide exposure increased susceptibility to and transmission of ranavirus. I 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. I also found that prior pesticide exposure exacerbated disease-induced mortality by increasing mortality rates, but effects on susceptibility to infection and transmission of the pathogen were minimal. Natural and anthropogenic stressors are common and regularly co-occur in natural systems, and as my results suggest, may have detrimental interactive effects on host species. These results underscore the importance of exploring these interactions and, in particular, addressing the order and timing of exposure to fully understand how stressors interact in a variable environment.

1.2 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 (Relyea and Hoverman 2006, Köhler and Triebkorn 2013). In non-target organisms, pesticides have been linked to endocrine disruption, developmental abnormalities, altered immune function, behavioral changes, and mortality (McKinlay et al. 2008, Hayes et al. 2010, Egea-Serrano et al. 2012, Gill et al. 2012, Brühl et al. 2013, Di Prisco et al. 2013, Mason et al. 2013). Moreover, changes that affect reproduction, survival, and species interactions have been implicated in trophic cascades in terrestrial and aquatic systems (Relyea et al. 2005, Cahill et al. 2008, Rohr et al. 2008b, Whitehorn et al. 2012, Beketov et al. 2013, Chiron et al. 2014, Hallmann et al. 2014). 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 (Koprivnikar 2010, Blaustein et al. 2011, O’Gorman et al. 2012, Goulson et al. 2015). 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 (De Castro and Bolker 2004, Smith et al. 2006, Johnson et al. 2015). These disease agents often comprise a substantial proportion of biomass in natural systems, perform important functions in trophic webs, and regulate host population sizes (Scott and Dobson 1989, Lafferty et al. 2006, Kuris et al. 2008). While infectious diseases are a natural component of communities, there is concern that environmental stressors may exacerbate disease outcomes (Smith et al. 2006, 2009). 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 Pietroock 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, Marcogliese et al. 2010, Di Prisco et al. 2013). These physiological changes have also lead to increased morbidity and mortality in host species, as seen in *Daphnia magna* and amphibians (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 (Tekwanl et al. 1988, Samanta et al. 2003, Ahmad and Srivastava 2007). 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, Rohr et al. 2008a, 2013, Koprivnikar 2010). 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 (Jancovich et al. 1997, Bollinger et al. 1999, Docherty et al. 2003). Moreover, they have been implicated in worldwide mass mortality events (Green et al. 2002, Fox et al. 2006, Une et al. 2009, Ariel 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 of these studies, since individuals were exposed to pesticide and ranavirus simultaneously. A simultaneous exposure complicates our ability to determine the driver of these interactive effects, as both stressors may be able to influence the other. Addressing specific orders and timings of exposure may elucidate how infection and pesticide exposure affect each other.

The objectives of my 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. I 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, I 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, I expected this to correlate with an increase in viral shedding rate and transmission to conspecifics.

1.3 Materials and Methods

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 hour day:night photoperiod) for 24 hours prior to the start of each experiment. Unless noted otherwise, during all experiments, water changes were conducted every 4 d and tadpoles were fed Tetramin *ad libitum* every 2 d.

Ranavirus was isolated from an infected larval green frog (*Lithobates clamitans*) collected from the Purdue Wildlife Area (PWA) in West Lafayette, IN. The virus was passaged through fathead minnow cells 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.

Pesticide application

I 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 (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, I 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. I 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 water. Nominal pesticide concentrations were verified at the Bindley Bioscience Center Metabolite Profiling Facility at Purdue University (Table 1.1).

Experiment 1 – Effects of ranavirus exposure on LC50 values

I performed LC50 tests to determine the effects of ranavirus exposure on pesticide toxicity estimates. My experiment was a randomized factorial design consisting of seven pesticide treatments and two virus treatments. The pesticide treatments consisted of a control (0 mg L⁻¹) and three concentrations (0.3, 3, and 30 mg L⁻¹) of each pesticide. The ranavirus treatments consisted of a no-virus control and exposure to ranavirus at a concentration of 10³ PFUs mL⁻¹. Experimental units were 2-L plastic tubs filled with 1 L

of filtered, UV-irradiated aged well water. I randomly assigned 10 tadpoles at Gosner stage 28 (Gosner 1960) to each unit. I replicated the 14 treatments four times for a total of 56 experimental units.

I first added 1.43 mL of the virus (original titer 7×10^5 PFUs mL⁻¹) to each virus treatment to achieve a final concentration of 10^3 PFUs mL⁻¹. Previous studies have demonstrated that this dosage is sufficient for initiating infection in wood frogs and other ranids (Hoverman et al. 2010, 2011). For instance, 95% infection prevalence was documented using identical exposure conditions (Hoverman et al. 2011). I 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. I chose to begin the LC50 test on day 4 of ranavirus exposure because I 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 for the LC50 test, providing 4 d to ensure infection and ending the experiment before the day 7 mortality spike would allow me to detect differences between exposed and unexposed individuals.

The LC50 tests were initiated on day 4 by randomly assigning experimental units from each virus treatment to the pesticide treatments. I 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 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.

Experiment 2 – Effects of pesticides on ranavirus susceptibility

To determine the effect of pesticide exposure on susceptibility to ranavirus, I conducted a randomized factorial experiment consisting of three pesticide treatments and three ranavirus treatments. The pesticide treatments consisted of a control (0 mg L⁻¹) and exposure to carbaryl (1 mg L⁻¹) or thiamethoxam (1 mg L⁻¹). These concentrations were

sublethal to tadpoles in my pilot studies. Ranavirus treatments consisted of a no-virus control, immediate exposure to ranavirus at a concentration of 10^3 PFUs mL^{-1} following pesticide exposure, and ranavirus exposure (10^3 PFUs 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. I randomly assigned 10 tadpoles at Gosner stage 29 (Gosner, 1960) to each unit. I replicated each treatment four times for a total of 36 experimental units.

I 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. 2008), and pesticide solutions were 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 treatment. Tadpoles in the immediate virus exposure treatment were exposed to virus immediately after pesticide exposure on day 8. I added 1.43 mL of the virus (original titer 7×10^5 PFUs mL^{-1}) to achieve a final concentration of 10^3 PFUs 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 PFUs mL^{-1}). 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, I extracted DNA using a DNeasy Blood and Tissue Kit (Qiagen) and stored at -80°C until qPCR analysis. To prevent cross contamination during necropsies, I soaked

all tools and surfaces in 10% bleach for 10 minutes and changed gloves between samples.

Experiment 3 – Effects of pesticides on ranavirus transmission

To determine the effect of pesticide exposure on the transmission of ranavirus, I 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 x 2 factorial design manipulating pesticide and ranavirus exposure on the focal tadpoles. The pesticide treatments consisted of a control (0 mg L⁻¹) 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 10³ PFUs mL⁻¹. I 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. I randomly assigned one focal tadpole to each experimental unit.

I exposed focal tadpoles to their respective pesticide treatment 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. I 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, I 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, I used a protocol adapted from R. Warne (unpublished protocol). 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.

Ranavirus testing

I used quantitative polymerase chain reaction (qPCR) to determine the viral load of each sample using the methods of Forson and Storfer (2006). 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 . I 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, I calculated viral load as viral copies ng^{-1} of DNA.

Statistical analyses

To compare LC50 values in experiment 1, I 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}). I calculated LC50 values for each cohort individually using probit analysis, which produced four replicate LC50 values for each virus treatment. I 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. To compare survival among pesticide treatments for individuals exposed to virus in experiment 2, I used a one-way ANOVA to compare mean time to death. To compare viral load among pesticide

treatments, I conducted a general linear mixed model with experimental unit as a random factor. For experiment 3, I assessed the effects of pesticide treatments on the mean viral load of focal and naïve tadpoles with one-way ANOVAs. The no-virus treatments were excluded from the analysis because no individuals were infected. In analyzing viral loads of the naïve tadpoles, I 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 $\alpha=0.05$.

1.4 Results

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. 1.1). LC50 estimates were 72% and 55% lower in the virus treatment for carbaryl and thiamethoxam, respectively, compared to the no-virus treatments. I 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 ($F_{2,30} = 1.27$, $p = 0.30$).

Experiment 2 – Effects of pesticides on ranavirus susceptibility

Time to death decreased (i.e. tadpoles died faster) when tadpoles were exposure to pesticides prior to ranavirus infection ($F_{2,9} = 3.76$, $p = 0.06$; Fig. 1.2). 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 ($p = 0.17$). Pesticide exposure did not influence infection prevalence (100% of tadpoles were infected in the ranavirus treatment) or viral load at time of death ($F_{2,9} = 0.21$, $p = 0.82$; Fig 1.4). When ranavirus exposure was delayed 2 wk following pesticide exposure, there was no effect of the pesticide treatments on time to death ($F_{2,9} = 1.02$, $p = 0.40$; Fig. 1.3), infection prevalence (100% of tadpoles were infected in the ranavirus treatment), or viral load at time of death ($F_{2,9} = 3.27$, $p = 0.08$). Furthermore, there was no difference in viral load between the immediate and delayed exposure regimes ($F_{1,18} = 2.03$, $p = 0.17$).

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. 1.4). All focal hosts exposed to ranavirus were infected with an average viral load of 75,892 viral copies ng DNA⁻¹. While I was 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. 1.5). 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.

1.5 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, Marcogliese et al. 2010, Di Prisco et al. 2013, Rohr et al. 2013). Moreover, research that addresses the effects of prior infection on estimates of pesticide toxicity is needed. I 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. I found that prior ranavirus infection can increase pesticide toxicity, and that pesticide exposure can alter disease outcomes.

I 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 (Green et al. 2002, Fox et al. 2006,

Une et al. 2009, Ariel 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. My results underscore the importance of considering scenarios in which pesticide exposure occurs following infection. Additionally, my 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.

I 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, I 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, I 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 have a high susceptibility and low tolerance to ranavirus infection, which may explain why there were no detectable differences in viral load. Given that 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 subtle changes in viral load by preventing individuals from reaching the high viral load threshold where they appear to experience mortality. Collectively, my 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, I examined the effects of pesticide exposure on ranavirus transmission. I 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. I 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 I was 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 my 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.

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 (Relyea 2012, Gergs et al. 2013, 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. I 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 I 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 coinfecting 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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Table 1.1 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

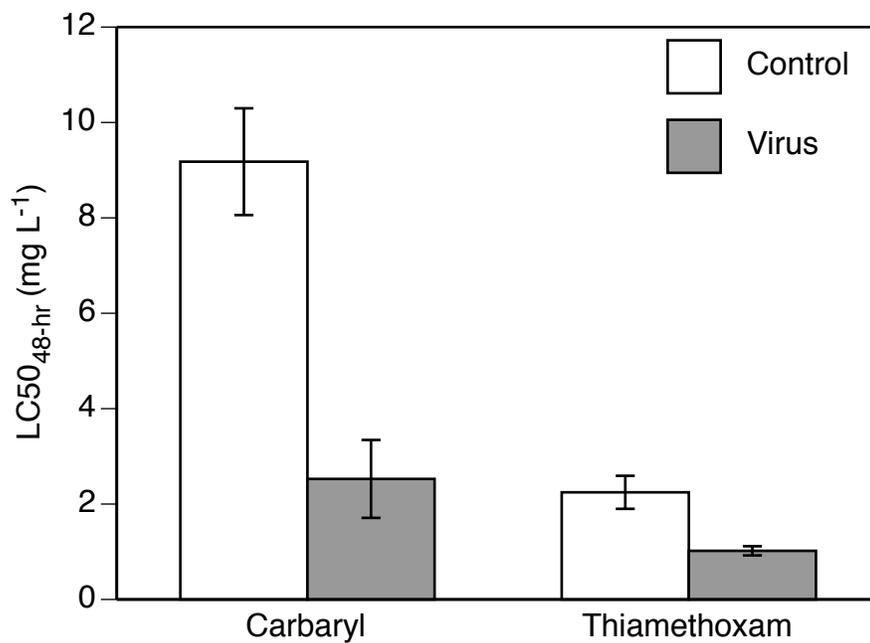


Figure 1.1 LC50_{48-hr} values for carbaryl and thiamethoxam for ranavirus-exposed and unexposed larval wood frogs. Data are means \pm 1 SE.

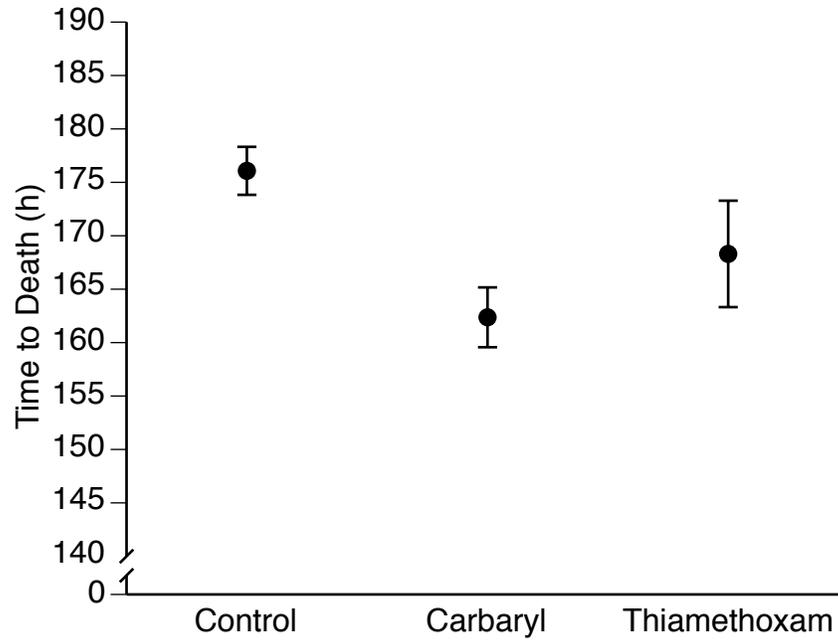


Figure 1.2 Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. Individuals were exposed to ranavirus immediately after pesticide exposure. Data are means \pm 1 SE.

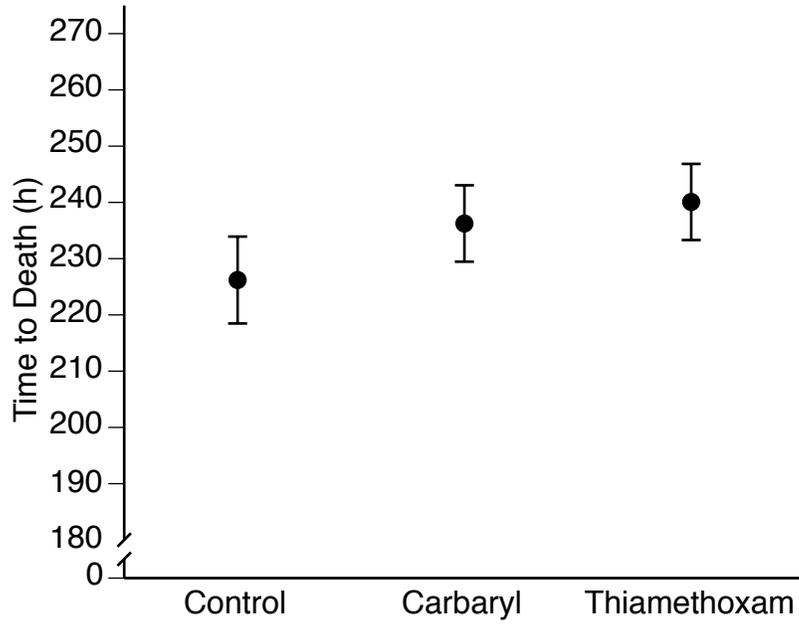


Figure 1.3 Time to death of ranavirus-exposed larval wood frogs across pesticide treatments. Individuals were exposed to ranavirus 2 wk after pesticide exposure. Data are means \pm 1 SE.

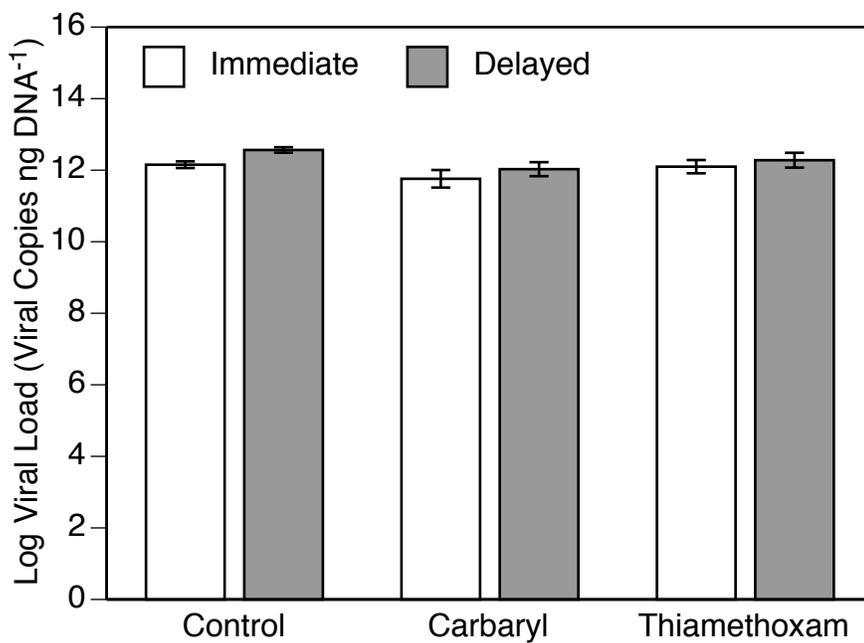


Figure 1.4 Viral load (viral copies ng DNA⁻¹) at time of death for ranavirus-exposed larval wood frogs that were previously exposed to no pesticides (control), carbaryl (1 mg L⁻¹) or thiamethoxam (1 mg L⁻¹). Individuals were either exposed to ranavirus immediately (“Immediate”) after pesticide exposure or 2 wk after pesticide exposure (“Delayed”). Data are means \pm 1 SE.

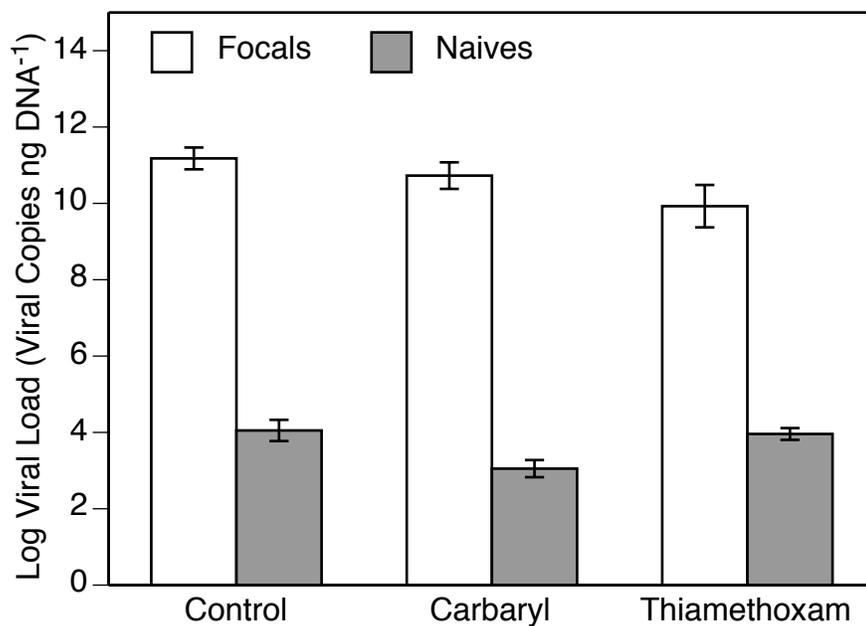


Figure 1.5 Viral load (viral copies ng DNA⁻¹) at time of death for ranavirus-infected focal and naïve larval wood frogs. Focal larvae were previously exposed to one of three insecticide treatments (a control, carbaryl at 1 mg L⁻¹, or thiamethoxam at 1 mg L⁻¹) before virus addition. Naïve larvae were not previously exposed to insecticides or ranavirus before addition to containers with water from focals. Data are means \pm 1 SE.

CHAPTER 2. PESTICIDES INFLUENCE THE TOLERANCE OF AMPHIBIAN HOSTS TO TREMATODE INFECTIONS

2.1 Abstract

Environmental contaminants such as pesticides are a growing threat to ecosystems because of their potential to cause direct mortality and indirectly influence species interactions. Given the recent emergence of infectious diseases in many species, research has increasingly focused on exploring the interaction between infectious disease and environmental contaminants. Across host-parasite systems, there is evidence that pesticide exposure increases parasite loads and mortality following infection. However, the mechanisms underlying these effects are often unclear. For instance, pesticide exposure could reduce the resistance of hosts to infection, slow the rate of parasite clearance once infected, or reduce the ability of the host to tolerate infection. Similarly, pesticide exposure could influence the parasite's ability to infect hosts and persist and reproduce within the host. Because of the large role that parasites play in natural systems, there is a need to understand how pesticide exposure of parasites affects their ability to successfully infect hosts. I examined how pesticides affect these mechanisms using larval northern leopard frogs (*Lithobates pipiens*) as hosts, the trematode *Echinoparyphium* sp., and the insecticide carbaryl. I found that pesticides did not affect the resistance mechanisms of behavioral avoidance or clearance in hosts. Moreover, there was no evidence that pesticide exposure of echinostomes altered infection success. However, in my analysis of tolerance, I found that pesticide exposure and parasite load have a negative interactive effect on host time to metamorphosis, causing earlier metamorphosis in more highly parasitized individuals by a factor of 31%. Interactive effects of pesticides and parasites on host life history have, to my knowledge, never been reported for the

amphibian-echinostome system. Given the routine co-occurrence of these pervasive stressors in natural systems and their potential for disrupting host life history, more research is needed to determine the implications of this interaction for ecological communities.

2.2 Introduction

Infectious disease is a central component of ecological communities, influencing host fitness, population dynamics, and community composition (De Castro and Bolker 2004, Smith et al. 2006, Johnson et al. 2015, Wood and Johnson 2015). Indeed, disease agents themselves comprise a substantial proportion of biomass in natural systems and perform important functions in trophic webs (Lafferty et al. 2006, Kuris et al. 2008). While disease research has often focused on host-pathogen interactions in isolation, there is an increasing interest in disease dynamics within the context of complex natural systems. In particular, the interaction of disease and environmental stressors such as climate change, habitat alteration, and chemical contamination are pertinent in a progressively human-influenced environment (Bradley and Altizer 2007, Rohr and Raffel 2010).

Contamination from pesticides is a stressor of particular concern due to the widespread use of pesticides on agricultural, commercial, and residential land. In the U.S., ~544 million kg of pesticides (active ingredient) are applied annually to a broad range of habitats (Grube et al. 2011). Moreover, these chemicals often enter natural systems, where they can affect non-target organisms (Relyea and Hoverman 2006, Grube et al. 2011, Marcogliese and Pietrock 2011, Mason et al. 2013, Köhler and Triebkorn 2013). In isolation, pesticide exposure has been shown to influence development, immune function, behavior, and survival (Egea-Serrano et al. 2012, Gill et al. 2012, Brühl et al. 2013, Di Prisco et al. 2013). Given the sublethal effects of pesticide exposure on host physiology, studies have increasingly explored the consequences for disease dynamics. Pesticide exposure has been associated with increased susceptibility to infection, greater pathology, and higher parasite abundance in communities (Christin et al. 2003, Coors et al. 2008, Rohr et al. 2008b, 2013, Di Prisco et al. 2013). While our understanding of this interaction is growing, there is a need to identify the mechanisms by which pesticide exposure influences disease. In particular, by distilling the effects of pesticides on the mechanisms by which hosts combat parasite damage and parasites combat host defenses, we can gain a clearer understanding of how pesticides affect host-parasite interactions.

When challenged with a parasite, hosts can either decrease their parasite load, a

process known as resistance, or they can decrease the damage caused by a certain parasite load, known as tolerance (Boots 2008, Read et al. 2008). Additionally, resistance can be further decomposed into parasite avoidance, where a potential host prevents infection, or parasite clearance, where a host's immune system will act to eliminate established parasites (Råberg et al. 2009). These processes are well understood in plant disease systems yet have only recently been incorporated into research on animals (Caldwell et al. 1958, Råberg et al. 2009). Given the highly variable environments in which hosts reside, it is likely that mechanisms of resistance and tolerance are affected by environmental conditions, including stressors. In particular, pesticide exposure is expected to alter these processes by disrupting immune function, causing physiological changes, and altering behavior (Marcogliese and Pietroock 2011). For example, pesticides have been shown to reduce leukocyte counts, which have been correlated with trematode clearance in amphibians (Kiesecker 2002, LaFonte and Johnson 2013). Pesticides have also been shown to decrease cholinesterase activity, leading to reduced movement in larval fish (Beauvais et al. 2001). Importantly, these mechanisms are context dependent, are subject to tradeoffs, and exert different selective pressures on hosts and parasites and may therefore be affected in different ways (Fineblum and Rausher 1995, Roy and Kirchner 2010, Rohr et al. 2010). For example, reductions in tolerance, but not resistance, to parasites have been demonstrated in yellow perch living in polluted environments (Marcogliese et al. 2010). Indeed, in some disease systems such as rodent malaria, resistance and tolerance mechanisms are found to be negatively correlated on a genetic level (Råberg et al. 2011). Ultimately, this research suggests that pesticide exposure may influence these mechanisms in a variety of ways; however a more comprehensive understanding of this interaction in different disease systems is needed.

While research suggests that pesticides can affect disease outcomes in hosts, less is known about the direct effects of pesticides on parasites. Free living parasites can experience mortality at environmentally relevant pesticide concentrations, and infectivity can be reduced by sublethal exposure (Koprivnikar et al. 2006, Rohr et al. 2008a, Hua et al. 2016). However, it is unclear whether reduction in infectivity is due to a hindered ability to invade and infect hosts or an inability to maintain infection when encountering

the host's immune system. Given the significant role parasites play in communities and ecosystems and their potential role as indicators of ecosystem health, it is important to consider how they are affected by environmental contaminants (Sures 2004, Lafferty et al. 2006, Kuris et al. 2008).

Amphibians are an ideal model system for studying pesticide-disease interactions due to the prevalence of pesticide contamination in wetland environments and the role of disease in driving their global population declines (Daszak et al. 2003, Relyea and Hoverman 2006). This interaction has frequently been studied using echinostomes, particularly *Echinostoma trivolvis* and *Echinoparyphium spp.* Echinostomes are widespread, highly prevalent parasites, which use larval amphibians as a second intermediate host in their life cycle (Kanev et al. 1995, Szuroczki and Richardson 2009). Free-swimming parasites enter larval amphibians through the cloaca and encyst in the kidneys, causing edema, decreased growth rates, and mortality (Schotthoefer et al. 2003, Szuroczki and Richardson 2009). Adult amphibians are often found with high parasite loads (> 2000 parasites), suggesting that parasite virulence may be fairly low (Johnson and McKenzie 2009). Nevertheless, host resistance and tolerance to echinostome infection has been documented and these mechanisms vary according to developmental stage and parasite species, indicating that they may be context-dependent (Rohr et al. 2010). Indeed, behavioral resistance mechanisms have been shown to vary with temperature, and parasite choice of hosts has been shown to vary with host mobility (Koprivnikar et al. 2006b, Johnson and Hoverman 2014). This suggests that other factors, such as pesticide exposure, may be influential as well. Indeed, host exposure to pesticides increases susceptibility to and mortality following echinostome infection (Budischak et al. 2008, Rohr et al. 2008a, Koprivnikar 2010). Pesticides also increase mortality and decrease infectivity in free swimming echinostomes (Koprivnikar et al. 2006, Rohr et al. 2008a, Hua et al. 2016). However, it is unclear how resistance and tolerance of amphibian hosts to infection is influenced by pesticide exposure. Additionally, research needs to address how pesticide exposure affects the ability of parasites to infect and maintain infections in hosts.

The objectives of my study were to determine whether pesticide exposure of amphibians affects behavioral resistance to echinostome infection, parasite host choice, tolerance of infection, and parasite clearance. I also sought to determine whether pesticide exposure of parasites affects infection success, including the ability to infect hosts and maintain infection over time. I expected that pesticide exposure would inhibit acetylcholinesterase activity, decreasing movement (Bridges 1997, Beauvais et al. 2001), reducing behavioral resistance, and altering host choice. Further, I hypothesized that pesticide exposure would increase host stress and metabolism, thereby reducing the ability to tolerate high parasite loads. If pesticide exposure causes immunosuppression (i.e. reduced leukocyte counts) in hosts, I expected to see a reduced clearance rate. Lastly, I hypothesized that parasites exposed to pesticides would have reduced initial infection success and increased clearance rates by hosts.

2.3 Materials and Methods

Species collection and husbandry

All experiments were carried out using northern leopard frogs (*Lithobates pipiens*) collected as 5 partial egg masses from a pond in West Lafayette, IN, USA on 5 April 2015. Egg masses were reared outdoors in 100-L pools filled with about 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 hour day:night photoperiod) for 24 hours prior to the start of each experiment.

To obtain *Echinoparyphium*, I collected their intermediate hosts, ramshorn snails (*Helisoma trivolvis*) from local ponds in West Lafayette, IN. The snails were screened for infection by placing individuals in 50-mL tubes filled with 45 mL of aged well water and allowing them to shed the free-living stage of the parasite (cercariae) under a heat lamp (Hua et al., 2016). Infected snails were housed in 15-L tubs filled with 8 L of aged well water at a density of 3 individuals L⁻¹ and fed rabbit chow *ad libitum* until the start of the experiments. To obtain *Echinoparyphium* cercariae for experiments, snails were induced to shed parasites as described above. I used a glass pipette and stereo dissection scope to

isolate and count cercariae for each experiment. The appropriate number of cercariae for each experiment was transferred to clean 50-mL tubes filled with 45 mL of aged well water and immediately added to the appropriate experiment unit. For treatments not assigned *Echinoparyphium*, I repeated this procedure adding the same volume of water from uninfected snails. The echinostomes used in these experiments were classified as *Echinoparyphium spp.* based on a genetic analysis of echinostomes concurrently collected from the same ponds (Hua et al. 2016).

Focal pesticide

For each experiment, I used the widespread insecticide carbaryl, an acetylcholinesterase inhibitor. Carbaryl is applied as an agricultural insecticide, with application rates reaching 400,000 kg annually and surface water concentrations measured as high as 4.8 mg L⁻¹ in the United States (Baker and Stone, 2015; Norris et al., 1983). LC50 estimates of carbaryl range from 7.4-9.6 mg L⁻¹ for northern leopard frogs and approximately 50 µg L⁻¹ for *Echinoparyphium* cercariae (Bridges et al., 2002; Hua et al., 2016). I chose sublethal, environmentally relevant concentrations for my experiments that were verified as sublethal using pilot studies. I created a working solution by adding 1 mL of commercial grade carbaryl (22.5% Sevin) to 9 mL of filtered, UV-irradiated water to achieve a concentration of 23,600 mg L⁻¹ of carbaryl. For each experiment, I used the working solution and filtered, UV-irradiated water to create the appropriate pesticide concentrations. Nominal pesticide concentrations were verified at the Bindley Bioscience Center Metabolite Profiling Facility at Purdue University.

Sample processing

For each individual, I measured weight, snout-vent length (SVL), total length, and developmental stage (Gosner 1960). I removed tadpole kidneys under a dissecting scope, placed them between two slides to create a thin layer of tissue, and counted metacercarial cysts (Hoverman et al., 2013). I also searched the remainder of the tadpole body cavity to ensure all cysts were counted.

Experiment 1 – Effects of pesticide exposure on resistance to Echinoparyphium cercariae

I used a randomized factorial experiment consisting of three pesticide treatments and two parasite treatments to examine the effects of pesticide exposure and presence of

cercariae on behavior and parasite resistance of tadpoles. Pesticide treatments consisted of a control (0 mg L⁻¹), exposure to carbaryl (1 mg L⁻¹) for 1 h followed immediately by exposure to cercariae, and exposure to carbaryl (1 mg L⁻¹) for 1 h followed by exposure to cercariae two days later. Tadpoles exposed to cercariae two days later were housed in fresh water for the 2-day interim. This delayed treatment was included to determine if behavior is altered after tadpoles have had the opportunity to metabolize the pesticide, as 48 h has been shown to be sufficient in ameliorating carbaryl-mediated reductions in cholinesterase activity (Beauvais et al. 2001). The actual pesticide concentration was 0.8 mg L⁻¹. The parasite treatments consisted of a control (0 cercariae) or exposure to 50 cercariae. I replicated the six treatments 10 times for a total of 60 experimental units. Experimental units consisted of 2-L containers filled with 1 L filtered, UV-irradiated water. Each unit housed one tadpole at Gosner (1960) stage 27 ± 0.048 with mass 0.13 ± 0.004 g (mean \pm SE). Tadpoles were exposed to their respective pesticide treatment followed by control or parasite treatment.

Immediately following exposure to the parasite treatment, tadpoles were monitored for movement (yes or no) at 10 s intervals for 5 min for a total of 30 observations (Koprivnikar et al. 2006). An individual was considered moving if it was swimming or conducting tail flicks during the 10 s interval. The response variable was the percent of time spent active (herein, activity), calculated as the percent of 10 s intervals in which the tadpole was active. After 15 min, tadpoles were moved to clean water for 24 h before being euthanized in MS-222 and preserved in 10% formalin for processing.

A two-way analysis of variance (ANOVA) was used to assess differences in activity among pesticide and parasite treatments. I arcsine-square-root transformed activity to meet the assumption of homoscedasticity. I included tadpole mass as a covariate because mass was correlated with behavior ($F_{1,53} = 4.452$, $p = 0.040$). I used an analysis of covariance (ANCOVA) to determine if activity and pesticide treatment influenced parasite load. All analyses herein and following were performed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA) at $\alpha = 0.05$.

Experiment 2 – Effects of pesticide exposure on the choice of hosts for Echinoparyphium cercariae

I examined whether pesticide exposure affects the distribution of *Echinoparyphium* between co-occurring hosts that differ in their pesticide exposure history. More specifically, I exposed one focal host to a pesticide treatment, paired it with a pesticide-naïve host in fresh water, and challenged the pair with cercariae. Focal and naïve hosts were marked with unique visual implant elastomer (VIE) tags 24 h prior to the start of the experiment. Focals were exposed to one of three pesticide treatments: (1) a control (0 mg L⁻¹), (2) exposure to carbaryl (1 mg L⁻¹) for 1 h immediately followed by parasite exposure, and (3) exposure to carbaryl (1 mg L⁻¹) for 1 h followed by parasite exposure two days later. The actual pesticide concentration was 0.8 mg L⁻¹. Naïve hosts were maintained in fresh water prior to the experiment. The three treatments were replicated 10 times for a total of 30 experimental units. Experimental units were 2-L tubs filled with 1 L fresh filtered, UV-irradiated water. Each pesticide-exposed and pesticide-naïve pair was exposed to 50 cercariae for 6 h and then moved to fresh water for an additional 18 h. Tadpoles were euthanized in MS-222 and preserved in 10% formalin for processing. I measured mass and determined developmental stage to ensure no differences between focal and naïve hosts. I tested whether the proportion of parasites that encysted between focal and naïve hosts differed from a 50% distribution using a two-tailed t-test.

Experiment 3 – Effects of pesticide exposure on tolerance to Echinoparyphium infection

I assessed whether pesticide exposure influences the tolerance of tadpoles to *Echinoparyphium* infection using a randomized factorial experiment consisting of two pesticide treatments and two parasite exposure treatments. Pesticide treatments consisted of a control (0 mg L⁻¹) or exposure to carbaryl (0.5 mg L⁻¹ added weekly). Parasite treatments consisted of a control (0 cercariae) or exposure to cercariae. Each treatment was replicated four times for a total of 16 experimental units and each experimental unit consisted of 20 tadpoles.

The experimental units were 1000-L outdoor cattle tanks filled with 500 L of well water. To each tank, I added 30 g of rabbit chow for an initial nutrient source, 300 g of oak leaf litter as a source of refugia and a surface for periphyton growth, and a 1-L aliquot of local pond water containing periphyton, phytoplankton, and zooplankton. I

covered tanks with 70% shade cloth to prevent colonization by undesirable insects and amphibians. The base community in the tanks was allowed to establish for 2 wk prior to the addition of tadpoles (Relyea and Hoverman, 2008).

I generated variation in parasite loads in order to determine a relationship between parasite load and measurements of fitness to serve as an estimate of tolerance. I generated this variation by exposing tadpoles to cercariae at low, medium, and high concentrations (33, 66, and 100 cercariae per tadpole) in the laboratory before moving them to the tanks. I marked tadpoles with VIE according to their assigned parasite load 24 h prior to the start of the experiment. I included control animals that were not exposed to parasites but marked with VIE to ensure no confounding effects of marking on fitness.

Echinoparyphium infection was conducted using group exposures wherein 35 marked tadpoles from each of the three parasite concentration groups were randomly assigned to one of two 15-L containers filled with 8 L filtered, UV-irradiated water. I obtained cercariae from infected snails and pooled the cercariae into a single container. After stirring the container to homogenize the distribution of cercariae, I haphazardly selected five 1-mL water samples to estimate the mean concentration of cercariae (mean = 11.4 mL⁻¹). Based on this concentration, I added 100, 200, and 300 mL of the water containing cercariae to the low, medium, and high tubs to achieve a final exposure of 33, 66, and 100 cercariae per tadpole. Tadpoles were maintained in containers for 24 h before being transferred to their respective experimental units. I randomly selected six tadpoles from each exposure dosage to include into experimental units. I added two additional randomly chosen tadpoles from across all exposure dosages for a total of 20 tadpoles per experiment unit.

Carbaryl was added to the appropriate experimental units just prior to the addition of tadpoles. Because carbaryl has a relatively short half-life (10 d at pH = 7; Sharom et al. 1980), I conducted weekly applications of the pesticide to ensure relatively consistent exposure. Immediately following pesticide exposure, carbaryl concentrations in the experiment units measured 0.08 mg L⁻¹. After one week, concentrations were reduced to 0.002 mg L⁻¹. The experiment was monitored weekly until individuals began to metamorphose. At this time, I monitored the tanks daily and removed individuals that

reached Gosner (1960) stage 42. These individuals were brought into the laboratory to complete metamorphosis in covered 15-L tubs filled with 1 L filtered, UV-irradiated water. Individuals were monitored daily and at Gosner (1960) stage 46 were euthanized in MS-222 and preserved in 10% formalin for processing. I recorded mass, length (SVL), and time to metamorphosis for each individual. To simulate ephemeral wetland drying, I gradually reduced the water level in each tank to 100 L over the course of 20 d. Water reduction began on August 4, 2015 and finished at the end of the experiment. All individuals that had not metamorphosed by the end of the experiment were treated as not surviving to metamorphosis.

I compared the proportion metamorphosed among pesticide and parasite treatments using a two-way ANOVA. I used general linear mixed models to determine the effects of parasite load and pesticide exposure on time to metamorphosis and mass at metamorphosis, two common measurements of amphibian fitness (Earl and Whiteman 2015). Experimental unit was included as a random factor in these models. For the model analyzing time to metamorphosis, the dependent variable was log transformed to meet the assumption of linearity and mass was used as a covariate ($F_{1,73} = 16.88$, $p < 0.001$).

Experiment 4 – Effects of pesticide exposure on clearance of Echinoparyphium

I performed a randomized 2 x 4 factorial experiment manipulating pesticide exposure and time since parasite exposure to determine the effects of pesticide exposure on the ability of tadpoles to clear *Echinoparyphium* infection. Pesticide treatments consisted of a control (0 mg L^{-1}) and carbaryl (1 mg L^{-1}) exposure with no solution renewal over the course of the experiment. The actual pesticide concentration was 0.8 mg L^{-1} . Tadpoles were sampled at 2, 6, 10, and 14 d after infection. The eight treatments were replicated 15 times for a total of 120 experimental units, each containing one tadpole. I also included three uninfected tadpoles for each post-infection time period to ensure no prior *Echinoparyphium* infection was present; there were no parasites detected in these individuals. Experimental units were 1-L containers with 500 mL filtered, UV-irradiated water. Tadpoles were exposed to 100 cercariae for 24 h before being transferred to new containers, where they were exposed to their respective pesticide treatments. Tadpoles were exposed to cercariae for 24 h to prevent behavioral resistance

and allow infection. Tadpoles were euthanized in MS-222 and preserved in 10% formalin for processing at each time point. I assessed if pesticide exposure and time since parasite exposure affected parasite load with a two-way ANOVA.

Experiment 5 – Effects of pesticide exposure on Echinoparyphium infection success

To determine whether pesticide exposure affects the ability of *Echinoparyphium* to successfully infect and maintain infection in tadpoles, I performed a randomized 2 x 4 factorial experiment manipulating pesticide exposure of parasites and time since infection. This experiment was designed nearly identically to Experiment 4 with the exception that the *Echinoparyphium* cercariae were exposed to pesticides, while the host tadpoles were unmanipulated. Pesticide treatments consisted of a control (0 mg L⁻¹) or carbaryl (0.05 mg L⁻¹) exposure for the parasites. The actual pesticide concentration was 0.02 mg L⁻¹. Tadpoles were sampled 2, 6, 10, and 14 d after infection. The eight treatments were replicated 15 times for a total of 120 experimental units. Experimental units were 2-L containers filled with 1 L filtered, UV-irradiated water and contained an individual tadpole. Tadpoles were exposed to either 50 pesticide-exposed or unexposed cercariae. I also included three uninfected tadpoles for each post-infection time period to ensure no prior *Echinoparyphium* infection; there were no parasites detected in these individuals.

Cercariae were shed, counted, and transferred to 50-mL tubes where they were exposed to 45 mL of their respective pesticide treatments for 1 h. Because cercariae were too small to be filtered from their pesticide solutions without damaging them, the entire contents of the 50-mL tubes were transferred to their respective experimental units. Water from the experimental units was sampled to determine the concentration of residual carbaryl; concentrations were found to be negligible at 0.003 mg L⁻¹. Tadpoles were exposed to cercariae for 1 h before being moved to fresh water. Exposure was limited to 1 h to allow for the effects of behavioral resistance, since I was interested in overall success of the parasite, including its ability to evade host resistance mechanisms. Tadpoles were euthanized in MS-222 and preserved in 10% formalin for processing at their respective time points. I used a two-way ANOVA to determine if pesticide exposure and time since infection influence parasite load.

2.4 Results

Experiment 1 – Effects of pesticide exposure on resistance to Echinoparyphium cercariae

There was no effect of pesticide exposure ($F_{2,53} = 0.572$, $p = 0.57$), parasite exposure ($F_{1,53} = 2.170$, $p = 0.15$), and their interaction ($F_{2,53} = 1.980$, $p = 0.15$; Fig. 2.1) on tadpole activity. However, there was a trend of higher activity in infected individuals from the carbaryl-initial treatment compared to the control ($p = 0.045$) and carbaryl-delayed treatment ($p = 0.093$). There was a marginal effect of activity on parasite load ($F_{1,24} = 3.51$, $p = 0.07$). However, there was no effect of pesticide exposure ($F_{2,24} = 0.91$, $p = 0.42$), or the pesticide-by-parasite interaction ($F_{2,24} = 0.27$, $p = 0.77$; Fig. 2.2) on parasite load following infection.

Experiment 2 – Effects of pesticide exposure on the choice of hosts for Echinoparyphium

The proportion of *Echinoparyphium* encysted in focal hosts did not differ from 0.5 in both the control and carbaryl-delayed exposure treatment (control, $t_9 = 1.94$, $p = 0.09$; carbaryl-delayed, $t_9 = -1.93$, $p = 0.09$). However, there was a trend for the proportion of *Echinoparyphium* encysted in focal hosts to be greater than 0.5 in the carbaryl-immediate exposure ($t_9 = 2.22$, $p = 0.053$; Fig. 2.3). Focal and naïve tadpoles did not differ in mass for the control group ($F_{1,18} = 2.12$, $p = 0.16$) or the carbaryl-immediate group ($F_{1,18} = 3.55$, $p = 0.08$). However, naïve tadpoles were larger than focal tadpoles in the carbaryl-delayed group ($F_{1,18} = 5.11$, $p = 0.04$).

Experiment 3 – Effects of pesticide exposure on tolerance to Echinoparyphium infection

I found no effect of pesticide exposure ($F_{1,12} = 1.19$, $p = 0.30$), parasite infection ($F_{1,12} = 0.50$, $p = 0.49$) or their interaction ($F_{1,12} = 0.33$, $p = 0.58$; Fig 2.4) on the proportion of individuals that survived to metamorphosis. In my analyses of the effects of pesticide and parasite load on fitness, parasite load ($F_{1,68} = 12.04$, $p = 0.001$) and the interaction of pesticide and parasite load ($F_{1,69} = 8.33$, $p = 0.005$; Fig. 2.5) were significant predictors of time to metamorphosis while pesticide alone had no effect ($F_{1,15} = 1.32$, $p = 0.27$). However, none of the predictors had a significant effect on mass at metamorphosis (pesticide, $F_{1,13} = 0.76$, $p = 0.40$; parasite load, $F_{1,69} = 0.62$, $p = 0.44$; pesticide*parasite load, $F_{1,69} = 2.72$, $p = 0.10$; Fig. 2.6).

Experiment 4 – Effects of pesticide exposure on clearance of Echinoparyphium

I found no effect of pesticide ($F_{1,100} = 0.22$, $p = 0.64$) or time since infection ($F_{3,100} = 0.07$, $p = 0.97$) on parasite load. There was a weakly significant interactive effect of pesticide and time since infection on parasite load ($F_{3,100} = 2.68$, $p = 0.051$; Fig. 2.7); however, this is not indicative of a clearance trend because parasite load was not reduced over time.

Experiment 5 – Effects of pesticide exposure on Echinoparyphium infection success

There was no effect of pesticide ($F_{1,107} = 0.26$, $p = 0.61$) or the interaction of pesticide and time since infection ($F_{3,107} = 1.90$, $p = 0.13$) on parasite load. There was a weakly significant effect of time since infection on parasite load ($F_{3,107} = 2.67$, $p = 0.051$; Fig. 2.7), but similarly to previous experiment, this was not indicative of a clearance effect because parasite load did not decrease over time.

2.5 Discussion

Pesticide exposure and infectious disease are two common stressors in natural systems. Because organisms often experience them simultaneously, there is a need to examine their interaction. While research on this interaction often addresses the effects of pesticide exposure on host susceptibility to parasitic infection, more information is needed on how pesticides affect the mechanisms of resistance and tolerance by which hosts increase their fitness when challenged with parasites. Additionally, given the significant roles parasites play in natural systems, it is important to understand how pesticides affect a parasite's ability to successfully infect its host, an often overlooked component in pesticide-disease research. Using an amphibian-echinostome host-parasite system, I tested whether carbaryl exposure influenced host resistance and tolerance of parasites, and whether pesticides influence the ability of parasites to infect and maintain infection in hosts. I found a negative relationship between parasite load and time to metamorphosis in amphibians exposed to carbaryl, indicating that pesticide exposure and parasitism can interact to alter host life history characteristics. However, I found that carbaryl exposure of host amphibians had no effect on either behavioral resistance or parasite clearance, and that exposure of echinostomes to carbaryl did not alter infection success.

Pesticide exposure did not affect host behavior, either in the presence or absence of parasites. However, comparisons among infected individuals suggest that carbaryl exposure immediately prior to parasite exposure may increase activity, indicating that pesticides may influence behavioral resistance. While there was a negative trend between tadpole movement and parasite load, indicating that behavioral resistance may be occurring, there was no evidence that pesticides influenced the relationship. Reductions in tadpole movement due to carbaryl have been documented at higher concentrations (3.5 mg L⁻¹; Bridges 1997), suggesting that the concentration used in my experiment (1 mg L⁻¹) may be too low to detect a relationship between pesticide exposure and behavioral resistance. Additionally, I saw no difference in parasite choice between pesticide-exposed and control tadpoles, further indicating that pesticide exposure did not alter the ability to resist infection. While differences in mass between paired tadpoles were detected, size has not been found to influence parasite aggregation in paired tadpoles (Johnson and Hoverman 2014).

Pesticide exposure may also influence the ability of hosts to limit the harm caused by parasites (i.e. tolerance; Råberg et al. 2009). I used two common measurements of fitness, time to metamorphosis and mass at metamorphosis, as surrogates for tolerance. I did not find evidence that pesticide exposure reduced tolerance defined as mass at metamorphosis. However, pesticide exposure influenced time to metamorphosis wherein higher parasite loads in pesticide-exposed hosts resulted in earlier metamorphosis. This suggests that pesticide exposure and parasite infection are interacting to alter host life history. Similar effects on amphibian development have been shown using predator cues, and these changes were found to be dependent on the environment and additional stressors (Laurila and Kujasalo 1999, Niecieza 2000, Relyea 2002, 2007). This is the first demonstration of changes in time to metamorphosis due to the interaction of parasite infection and pesticide exposure. There is some debate whether time to and mass at metamorphosis are appropriate measures of fitness because these factors ostensibly do not have a strong influence on post-metamorphic fitness (Earl and Whiteman 2015, Schmidt et al. 2016). While no alternative surrogates of fitness have been proposed, my results indicate that pesticide exposure may affect parasite tolerance. Alternatively,

reduced time to metamorphosis may be a result of pesticide and disease-induced stress, as accelerated metamorphosis has been shown for amphibians infected with other disease agents and may be mediated by corticosterone levels (Romansic et al. 2011, Warne et al. 2011). Regardless, these results show an interactive effect of pesticides and disease on host life history, which may indicate an alteration in host tolerance to infection. In addition to estimating tolerance in individuals, I also analyzed how pesticide exposure and parasite infection affect overall survival to metamorphosis. I found no effect of carbaryl or parasites on survival to metamorphosis. While there is evidence that pesticide exposure and echinostome infection can increase mortality, this has only been shown using the herbicide atrazine (Koprivnikar 2010). Most studies focusing on insecticides have not demonstrated mortality effects (Budischak et al. 2008).

My analyses of clearance and parasite success showed no evidence of parasite clearance over time and no effect of pesticide exposure on clearance or infection. While clearance of trematodes has been documented for both echinostomes and the more debilitating *Riberoia ondatrae*, resistance has been found to be stronger for *R. ondatrae*, presumably because of its subcutaneous encystment and high virulence (LaFonte and Johnson 2013). In contrast, echinostomes exhibit a relatively low virulence, causing negligible fitness costs at moderate parasite loads (Orlofske et al. 2009). Additionally, host phylogeny has been found to be an important factor in determining parasite clearance rate (LaFonte and Johnson 2013). No previous studies on echinostome clearance have used ranids as a focal taxon. Therefore, differences among host taxa and low parasite virulence may explain why clearance was not detected. Echinostomes may also exhibit population-level variability in pesticide tolerance, further complicating the ability to measure the effects of pesticides on parasite success (Hua et al. 2016).

I found that pesticide exposure does not affect mechanisms of behavioral resistance or clearance in host amphibians challenged with echinostome parasites. Moreover, pesticide exposure of echinostomes does not affect infection success. Mechanisms of parasite resistance have been found to be highly context-dependent, changing with host species, parasite species, and environmental factors (Koprivnikar et al. 2006b, LaFonte and Johnson 2013, Johnson and Hoverman 2014). Additionally, stressors

can have different effects on resistance and tolerance and these two mechanisms may be inversely correlated in some species (Marcogliese et al. 2010, Raberg et al. 2011). Additional studies focusing on a broader range of host and parasite species and pesticides are needed for a more complete understanding of how pesticides might affect resistance and infection success. I found that pesticide exposure and parasite load do not have an interactive effect on mass at metamorphosis, but do affect time to metamorphosis, with individuals metamorphosing earlier with higher parasite loads when exposed to carbaryl. The interactive effects of pesticides and parasites on life history have never been documented for the amphibian-echinostome system. Given the ubiquity and regular co-occurrence of these stressors in natural systems, disruptions such as this may prove influential to host populations; however considerable research is needed on the effects of earlier metamorphosis on populations to understand the impacts of this effect.

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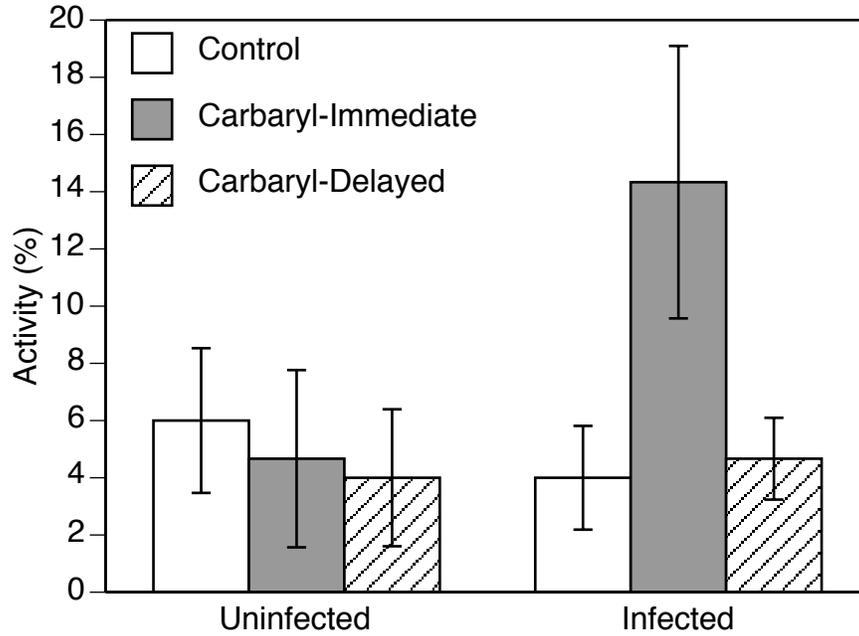


Figure 2.1 Activity (percent of time spent active) of larval northern leopard frogs across pesticide and parasite treatments. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed). Data are means \pm 1 SE.

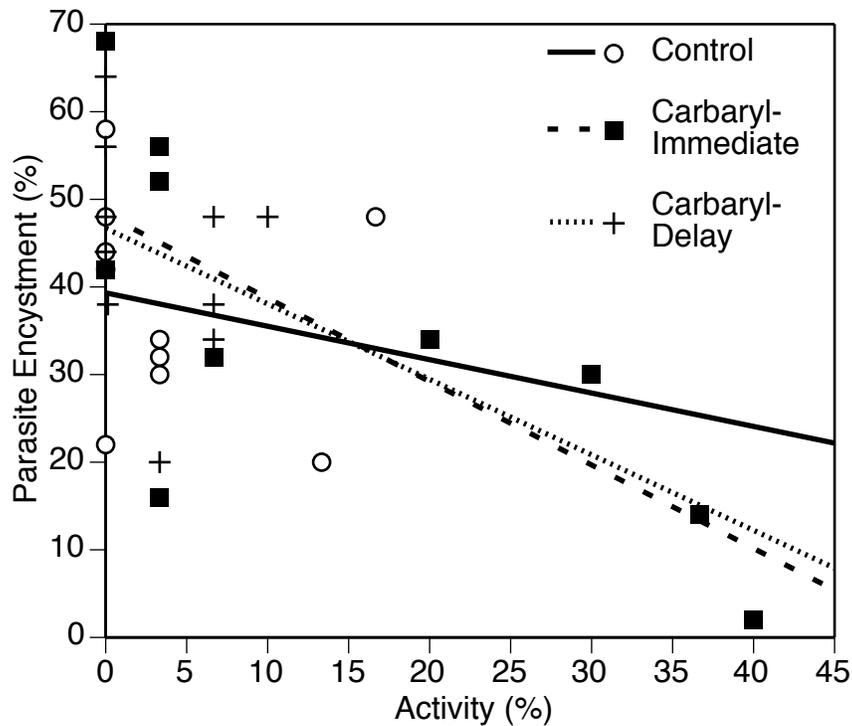


Figure 2.2 Relationship between parasite encystment (percent of the initial exposure amount) and activity (percent of time spent active) of larval northern leopard frogs across pesticide treatments. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed).

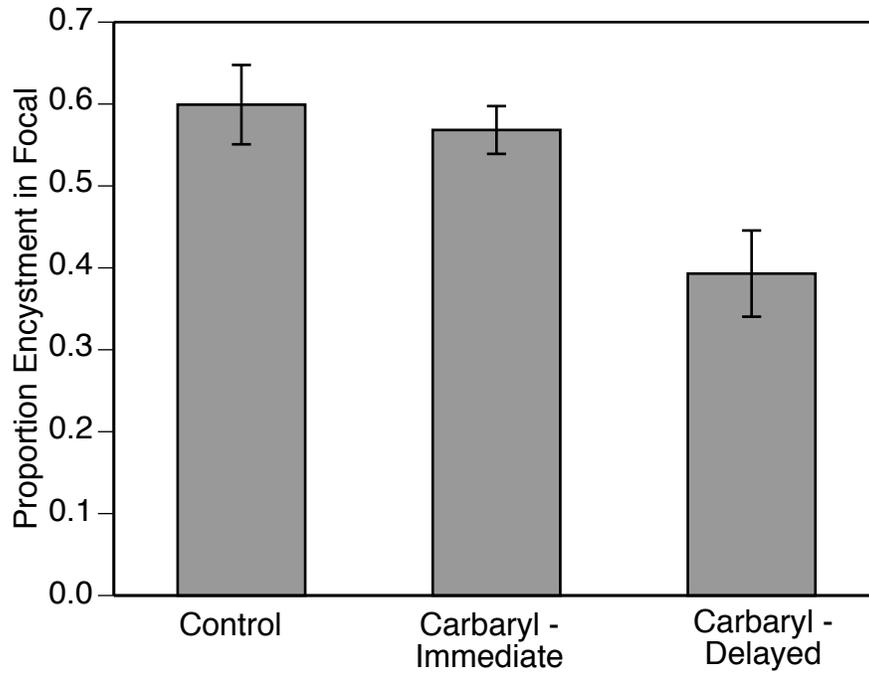


Figure 2.3 Proportion of parasites encysting in focal larval northern leopard frogs across pesticide treatment. Tadpoles were either exposed to parasites immediately following pesticide treatment (Carbaryl-Immediate) or 2 d following pesticide treatment (Carbaryl-Delayed). Data are means \pm 1 SE.

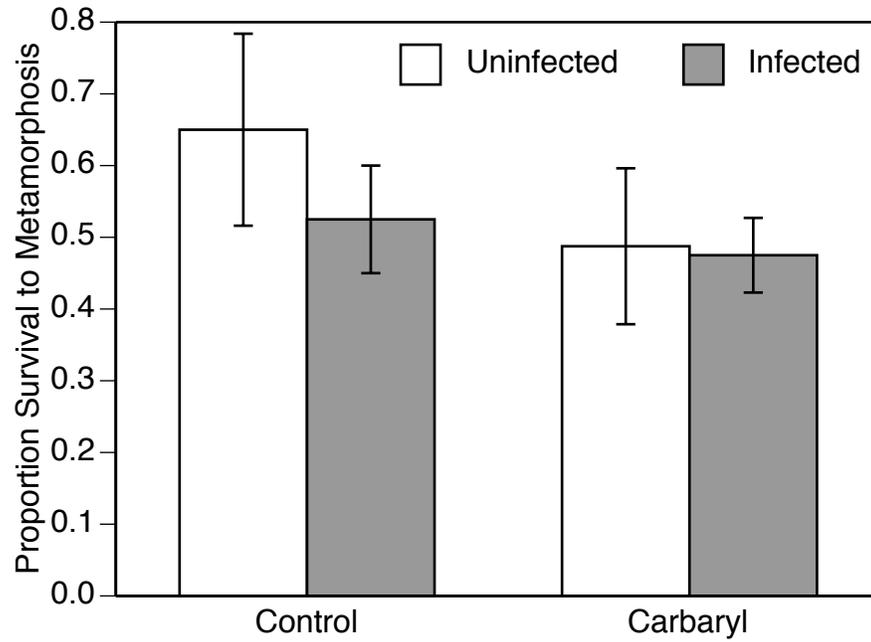


Figure 2.4 Proportion of northern leopard frogs surviving to metamorphosis within experimental tanks across pesticide and parasite treatments. Data are means \pm 1 SE.

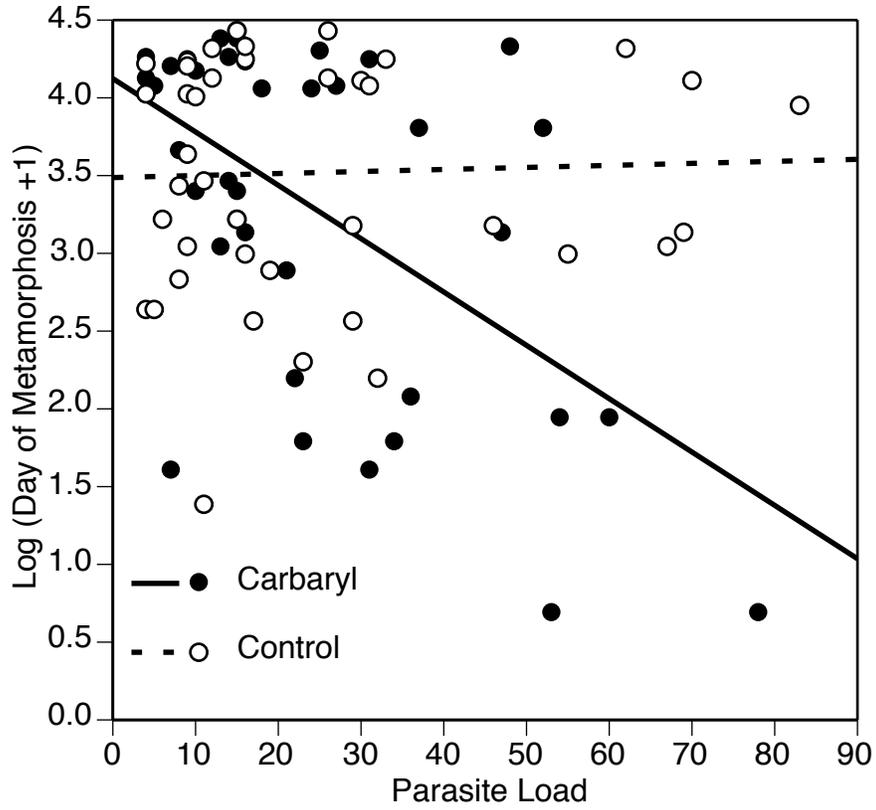


Figure 2.5 Relationship between parasite load and day of metamorphosis for larval northern leopard frogs across pesticide treatments. Day of metamorphosis was log transformed +1 to meet the assumption of linearity.

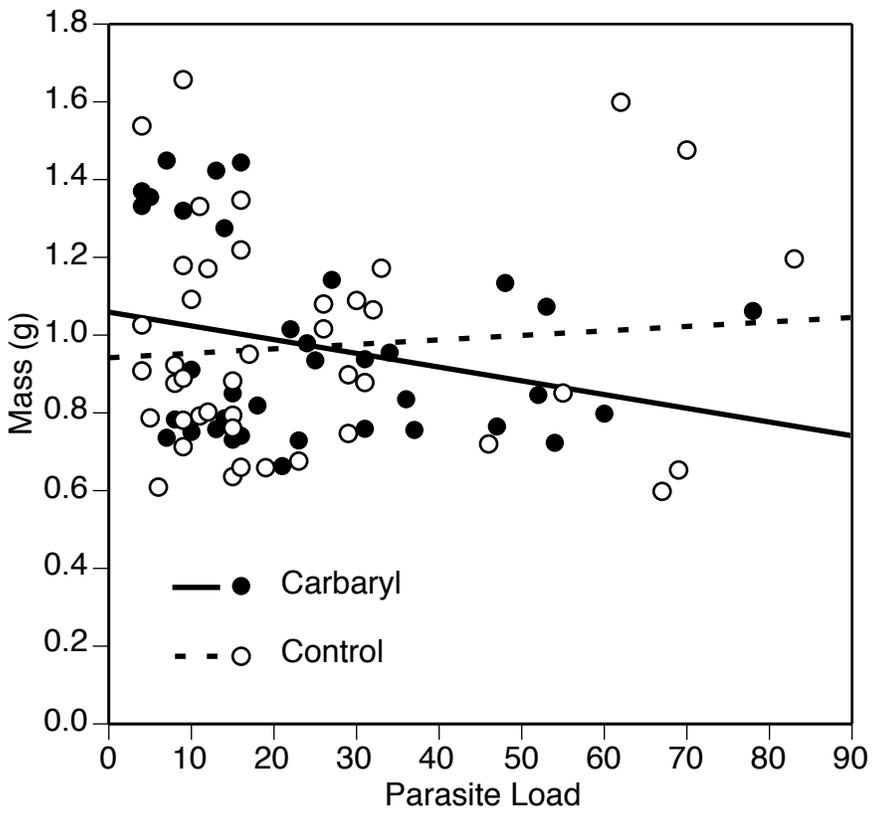


Figure 2.6 Relationship between parasite load and mass at metamorphosis for larval northern leopard frogs across pesticide treatments.

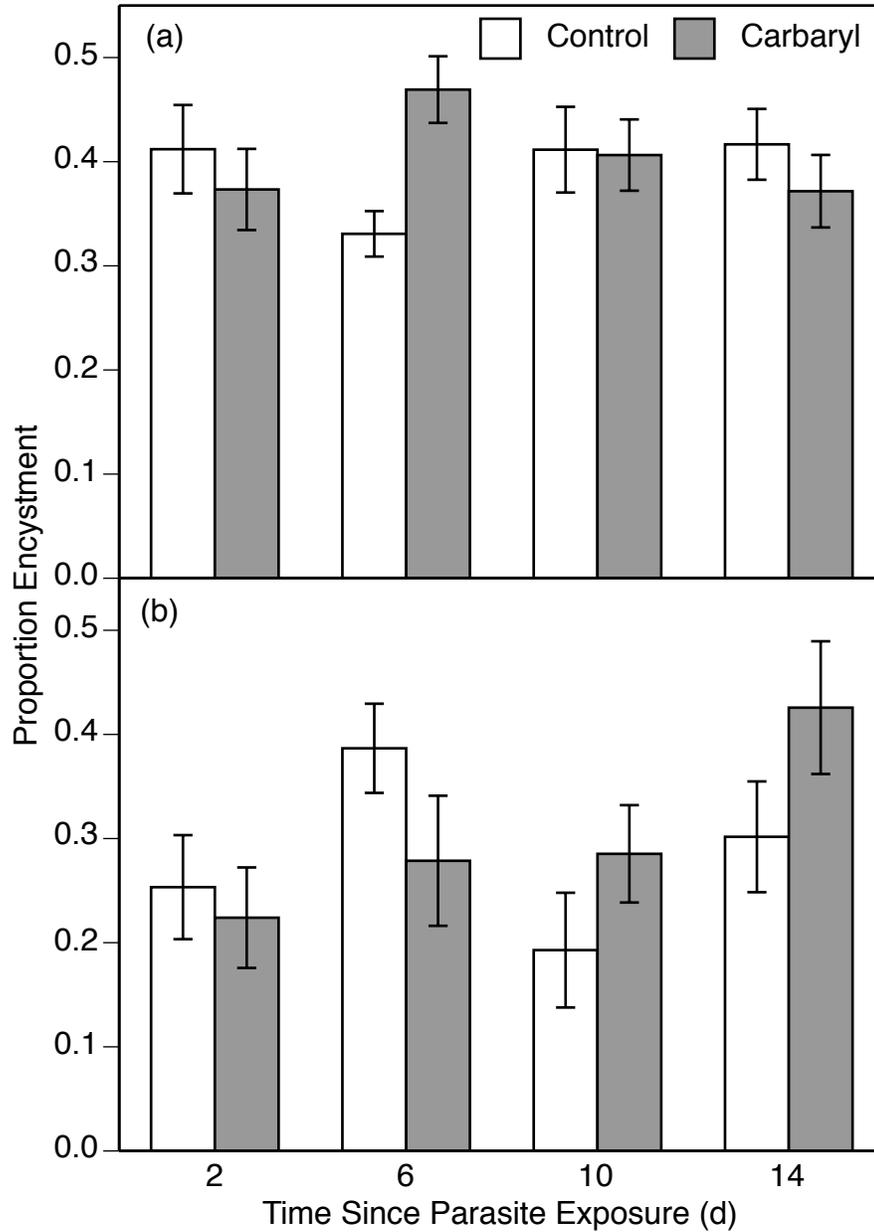


Figure 2.7 Proportion of parasites encysting in larval northern leopard frogs across time points and pesticide treatment. (a) Larvae were exposed to pesticide prior to being infected with *Echinoparyphium cercariae*. (b) *Echinoparyphium cercariae* were exposed to pesticide prior to infecting larvae. Data are means \pm 1 SE.

CHAPTER 3. CONCLUSIONS

3.1 Conclusions and Future Directions

Pesticide exposure and infectious disease are two common, regularly co-occurring stressors in natural systems. While the isolated effects of each stressor on ecological communities are well understood, less is known about their interactive effects. I conducted several studies to explore these interactive effects using amphibians as hosts and ranavirus and echinostomes as disease agents. I found that prior ranavirus infection increases the toxicity of pesticides, while prior pesticide exposure increases ranavirus-induced mortality, an effect that is ameliorated when individuals are allowed to metabolize the pesticide. Pesticides were found to have minimal effect on ranavirus transmission. Pesticide exposure did not alter resistance to echinostome infection, but reduced host time to metamorphosis at high parasite loads, potentially altering host tolerance of infection. In brief, my results indicate that pesticides and infectious disease have interactive effects on amphibian hosts; however, these effects vary based on order and timing of exposure and have a more pronounced influence over certain aspects of disease dynamics than others.

Several changes can be made to improve upon the methods used in my studies. First, wood frogs are a species that have a high susceptibility and low tolerance to ranavirus infection (Hoverman et al. 2011). They were chosen to ensure that a sufficient number of individuals became infected; however, their high susceptibility may be the reason that no differences in infection or viral load were detected. Additionally, a standard viral concentration of 10^3 PFUs mL⁻¹ was used to ensure infection, yet this concentration may be too high, leading to 100% infection and a rapid *in vivo* viral replication that limited my ability to detect differences in viral load. In my

examination of transmission, I was unable to detect virus in the water despite transmission clearly occurring. Here, using a smaller volume of water may make viral concentrations more detectable. In my study of pesticide-echinostome interactions, I found minimal effects on resistance or tolerance mechanisms, which may indicate that the parasite used, *Echinoparyphium*, is not virulent enough in the numbers used to elicit a response from the host (Orlofske et al. 2009). Using a more virulent species, such as *Riberoia ondatrae* may prove more effective for this study (LaFonte and Johnson 2013). Additionally, in my study of infection tolerance, the low density of tadpoles in experimental units (n = 20) may explain why no differences in mass at metamorphosis were observed, despite the difference observed in time to metamorphosis. Higher densities may allow an effect to be seen.

There are several future studies that can expand upon this research and resolve its limitations. Research on pesticide-ranavirus interactions is needed involving multiple amphibian species, particularly given the considerable phylogenetic variation in susceptibility to infection (Hoverman et al. 2011). My results indicating that infection increases pesticide toxicity also require further research involving additional species across host taxa. A better understanding of ranavirus transmission is also needed, particularly addressing the relationship between host viral load, shedding rate, and transmission to conspecifics. Finally, a clearer assessment of the consequences of altered time to metamorphosis on post-metamorphic fitness is necessary for understanding how pesticides might alter tolerance to infection in amphibians. By resolving these limitations, we can form a more comprehensive understanding of pesticide-disease interactions and the implications for ecological communities.

3.2 Literature Cited

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