EXPLORING THE SEPARATE AND INTERACTIVE EFFECTS OF PESTICIDES AND PARASITES ON AMPHIBIANS

by

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ABSTRACT

In the Anthropocene, amphibians must not only cope with natural stressors but also a suite of human-made stressors that have been experienced relatively recently within their evolutionary history. Because it has become increasingly common for natural and anthropogenic stressors to co-occur in aquatic ecosystems, the study of their separate and combined effects on ecosystems and their component species is increasingly necessary. This is especially important for amphibians, which have experienced global declines and can be highly sensitive to both natural and anthropogenic stressors. Pesticides and parasites are two commonly co-occurring stressors that can have complex individual and synergistic detrimental effects in amphibian populations. Here, I conducted three studies to broadly assess the separate and interactive effects of pesticides and parasites on amphibians. More specifically, I explored: 1) the underlying physiological mechanism allowing amphibians to induce increased tolerance to a pesticide within a single generation, 2) the effects of exposure timing to two functionally similar cryptic parasite species on infection outcomes, and 3) population-level variation in susceptibility to parasites and whether prior exposure to pesticides influenced the outcome of host-parasite interactions. First, to test the hypothesis that induced pesticide tolerance is the result of a generalized stress response, I exposed tadpoles to an anthropogenic stressor (sublethal pesticide dose), a natural stressor (cues from a caged predator), or a simulated stressor via exogenous exposure to the stress hormone corticosterone (CORT). I then exposed the larvae to a lethal carbaryl treatment to assess how the stressor exposures influenced survival. I found that prior exposure to exogenous CORT and predator cues induced tolerance to a lethal concentration of carbaryl, providing evidence that pesticide tolerance can be induced by a generalized stress response both in the presence and absence (exogenous CORT) of specific cues. Second, I explored how the timing of

host exposure to two co-occurring cryptic echinostome species influences infection outcomes. I found that echinostome infection success in larval anurans can differ significantly based on the parasite species makeup, density, and exposure timing. I also found that priority effects can occur even between functionally similar cryptic species, with an early exposure to *Echinoparyphium* lineage 3 reducing the infection success of *Echinostoma trivolvis* three days later. Finally, I assessed the influence of pesticide exposure on host-parasite interactions and population-level variation in these responses. This was accomplished by exposing wood frog larvae from eight populations to one of two treatments (a sublethal carbaryl concentration or a pesticide-free control) followed by controlled parasite exposures to either echinostome trematodes or ranavirus. Then, I assessed how pesticide exposure influenced infection loads, infection prevalence, and survival in each population. I found significant population-level variation in infection outcomes. Interestingly, however, I found no significant effects of pesticide exposure on disease outcomes. Together, these three studies demonstrate the wide-ranging and surprising outcomes that can result from interactions among and between natural and anthropogenic stressors.

CHAPTER 1. PESTICIDE TOLERANCE INDUCED BY A GENERALIZED STRESS RESPONSE IN WOOD FROGS (*RANA SYLVATICA*)

1.1 Abstract

Increasing evidence suggests that phenotypic plasticity can play a critical role in ecotoxicology. More specifically, induced pesticide tolerance, in which populations exposed to a contaminant show increased tolerance to the contaminant later, has been documented in multiple taxa. However, the physiological mechanisms of induced tolerance remain unclear. I hypothesized that induced pesticide tolerance is the result of a generalized stress response based on previous studies showing that both natural stressors and anthropogenic stressors can induce tolerance to pesticides. I tested this hypothesis by first exposing larval wood frogs (*Rana sylvatica*) to either an anthropogenic stressor (sublethal carbaryl concentration), a natural stressor (cues from a caged predator), or a simulated stressor via exogenous exposure to the stress hormone corticosterone (62.5 nM or 125 nM). I also included treatments that inhibited corticosterone synthesis with the compound metyrapone (MTP). I then exposed the larvae to a lethal carbaryl treatment to assess time to death. I found that prior exposure to both 62.5 and 125 nM of exogenous CORT induced tolerance to a lethal concentration of carbaryl. Similarly, tadpoles exposed to predator cues expressed induced tolerance to the lethal carbaryl concentration. Pre-exposure to sublethal carbaryl, as well as MTP alone or in combination with predator cues, did not induce tolerance to the lethal carbaryl concentration. This study provides evidence that pesticide tolerance can be induced by a generalized stress response both in the presence and absence (exogenous CORT) of specific cues and highlights the importance of considering physiological ecology and environmental context in ecotoxicology.

1.2 Introduction

As human populations continue to grow, the effects of anthropogenic stressors such as climate change, habitat destruction, and chemical contaminants on wildlife are becoming more pronounced (Woodward et al. 2010, Corlett 2015, Haddad et al. 2015, Bernhardt et al. 2017). There is growing evidence that globally ubiquitous chemical contaminants can have wide-ranging negative effects on individuals, communities, and ecosystems (Köhler and Triebskorn 2013). Pesticides are a particular concern because of their toxicity, environmental persistence, and heavy use in agriculture and pest control (Relyea and Hoverman 2006, Kashiwagi et al. 2009, Köhler and Triebskorn 2013). Although pesticide applications are typically targeted, these chemicals often leave their application site and enter surrounding ecosystems through processes such as runoff, drift, and leaching (Schriever and Liess 2007, Köhler and Triebskorn 2013). As a result, non-target wildlife species are often inadvertently exposed to pesticides in their natural environments (Gilliom et al. 2007, Schriever and Liess 2007, Relyea 2009).

There is accumulating evidence that populations that are frequently exposed to pesticides can evolve increased tolerance via natural selection (Denholm and Rowland 1992, Jasieniuk et al. 1996, Liu 2015). Though natural selection on constitutive traits over multiple generations is the traditional paradigm under which increased contaminant tolerance is predicted to occur (Brausch and Smith 2009, Futuyma and Kirkpatrick 2017), recent evidence suggests that some organisms can rapidly respond to anthropogenic environmental changes via another mechanism, phenotypic plasticity (Hua et al. 2013, 2015b, Oziolor et al. 2017, Wuerthner et al. 2019). Plasticity is the ability of a single genotype to produce multiple phenotypes under different environmental conditions (Schlichting and Pigliucci 1998, Pigliucci 2001) and includes changes in morphology (Hoverman and Relyea 2009), behavior (Sih et al. 2011), and physiological processes (e.g., detoxification pathway activity; Oziolor et al. 2017). Over the last decade,

studies with fish, amphibians, and invertebrates have documented the existence of induced pesticide tolerance, in which populations previously exposed to a sublethal pesticide concentration show increased tolerance to lethal concentrations days later (Poupardin et al. 2008, Hua et al. 2013, Jones and Relyea 2015, Oziolor et al. 2017, Wuerthner et al. 2019). Collectively, these studies suggest that induced pesticide tolerance could be a generalizable phenomenon that improves survival following exposure to chemical contaminants.

Generalized physiological mechanisms, such as the stress response, can lead to increased tolerance to a wide array of stressors (Snell-Rood et al. 2018). One of the main pathways by which vertebrates respond to stressors in the environment is the hypothalamus-pituitary-adrenal/interrenal (HPA/HPI) axis, which leads to increased production of glucocorticoids when activated (Denver 2009a). Glucocorticoids play a role in many physiological and behavioral processes including metabolic regulation, growth, immune responses, anti-predator responses, and detoxification pathway activity (Denver 2009b, Middlemis-Maher et al. 2013, Konstandi et al. 2014, Cain and Cidlowski 2017). Moreover, the increased production of glucocorticoids in the face of a stressor can shift energy allocation to physiological processes that play a key role in survival and potentially lead to tolerance to a wide array of stressors and contaminants (Sapolsky et al. 2000, Snell-Rood et al. 2018). Because the generalized stress response mediated by HPA/HPI activation is well conserved across vertebrate taxa, it represents a potential mechanism underlying induced pesticide tolerance.

In support of this line of inquiry, a recent study showed that previous exposure to naturally occurring stressors (i.e. predator cues from dragonfly larvae) induced pesticide tolerance in wood frog tadpoles (Jones et al. 2017). This suggests that in some situations, a stress response prior to exposure to a novel contaminant may have a preparative effect, or an effect that

mediates the individual's response to a later stressor (Sapolsky et al. 2000). Indeed, it has been documented that levels of the stress hormone corticosterone (CORT), a major glucocorticoid and part of the stress response in amphibians, are increased by the presence of predators (Middlemis-Maher et al. 2013). CORT levels can also be increased by the presence of pesticides; however, the response can be non-linear and dependent on both the mode of action and concentration of the pesticide (McMahon et al. 2017, Gabor et al. 2018). Collectively, this research suggests that: 1) CORT may mediate responses to both natural and anthropogenic stressors and 2) exposures to stressors early in life may indirectly alter future responses to contaminants. However, it remains unclear whether the stress response (via HPA/HPI), which is activated by a variety of stressors, plays an important role in induced pesticide tolerance.

My objective was to determine if induced pesticide tolerance can result from a generalized stress response. More specifically, I assessed if exposure to a simulated stressor (exogenous CORT), a natural stressor (predation risk), and an anthropogenic stressor (sublethal pesticide) induce pesticide tolerance in larval wood frogs. To provide additional evidence that the stress response induced tolerance and not some other response to the stress cues, I included treatments with the compound metyrapone (MTP), which inhibits corticosterone synthesis. After larval wood frogs were exposed to the pre-treatments, they were exposed to a lethal concentration of carbaryl to assess relative pesticide tolerance via time to death assays. If a generalized stress response has a preparative effect that increases pesticide tolerance, I predicted that exposure to exogenous stress hormone, a natural stressor, and an anthropogenic stressor would all result in induced pesticide tolerance in larval wood frogs. Likewise, I predicted that exposure to metyrapone and metyrapone in combination with a natural stressor or an

anthropogenic stressor would not result in induced pesticide tolerance due to the lack of a preparative stress response prior to pesticide exposure.

1.3 Methods

Insecticide Background

I chose the carbamate insecticide carbaryl (commercial formulation Sevin©, 22.5% active ingredient, CAS 63-25-2) as the focal pesticide for this study. First registered in 1959, carbaryl is a reversible acetylcholine esterase inhibitor (Čolović et al. 2013). Carbaryl has been heavily used for agricultural pest control and is still commonly used (1-1.8 million kilograms per year) for pest control in the residential sector (Atwood and Paisley-Jones 2017). Carbaryl has a half-life of 10 days at a pH of 7 and has been found at concentrations as high as 1.5 mg/L in aquatic environments (Norris et al. 1983). Many amphibian species, including wood frogs, are sensitive to carbaryl, with reported LC50 values ranging from 1.2 to 22 mg/L (Boone and Bridges 1999, Bridges and Semlitsch 2000, Relyea 2003).

Animal collection and husbandry

On 7 April 2018, we collected 10 newly oviposited wood frog egg masses from a pond in Crawford County, Pennsylvania (41.87° N, 80.47° W). This pond is located >500 m from any agricultural field, which makes frequent exposure to pesticides unlikely (Declerck et al. 2006). Additionally, studies have demonstrated that wood frog tadpoles from this population display increased tolerance to carbaryl following a sublethal exposure, making it an ideal focal population for this study (Hua et al. 2015b). Following collection, egg masses were immediately transported to the Purdue Wildlife Area (PWA) in West Lafayette, IN, and placed in 180-L outdoor culturing pools filled with aged well water for rearing. All tadpoles reached Gosner stage 25 by 30 April.

Experimental design

I conducted the experiment at the indoor facility at the PWA. On 1 May, 500 haphazardly selected hatchling tadpoles (mean mass ± 1 SE = 30 ± 1 mg; median Gosner stage = 25) were brought inside and placed into 15-L plastic tubs (n = 25 per tub) filled with 10 L of UV-irradiated, aged well water for 24-h acclimation to indoor conditions (23 °C, 12:12 light cycle).

I utilized a two-phase design similar to those used by Hua et al. (2013) and Jones et al. (2017) (Fig. 1.1). In phase 1, I exposed tadpoles to a vehicle control (no stressor), sublethal pesticide treatment, predator-cue treatment, one of two concentrations of the stress hormone corticosterone (CORT), the corticosterone synthesis inhibitor metyrapone (MTP), sublethal pesticide treatment + MTP, or predator-cue treatment + MTP. In phase 2, tadpoles from the phase 1 treatments were placed in one of two treatments: a no-insecticide control or a lethal insecticide treatment. I then conducted time to death (TTD) assays to determine how exposure to the phase 1 treatments influenced pesticide tolerance.

Phase 1: Sublethal Stressor Exposure

On 2 May, I haphazardly selected tadpoles and assigned them to one of the eight pre-treatments: a vehicle control, 0.5 mg/L carbaryl, predator cue, 62.5 nM CORT, 125 nM CORT, 0.5 mg/L carbaryl + 110 μM MTP, or predator cue + 110 110 μM MTP (Fig. 1.1). The experimental units were 68-L plastic tubs filled with 30 L UV-irradiated, aged well water. I conducted group exposures such that one tub was assigned to a treatment and contained 60 tadpoles. For the sublethal carbaryl exposure treatment, I selected 0.5 mg/L as the nominal carbaryl concentration. This concentration has been shown to induce tolerance in wood frog tadpoles in previous studies and is environmentally realistic (Norris et al. 1983, Jones et al. 2017). Although the effects of carbaryl on amphibian CORT levels have not been studied

explicitly, various pesticides at environmentally relevant concentrations have been shown to alter stress hormone levels in tadpoles (McMahon et al. 2011, 2017). For the predator cue treatment, I placed two caged tadpole predators (dragonfly larvae, *Anax junius*) individually in 1-L cups covered with mesh within the respective experimental unit. These cages allow predator cues to enter the water while preventing the predators from consuming the focal animals. Stress hormones play a role in anti-predator responses of tadpoles, and the presence of caged predators has been found to elevate corticosterone levels in tadpoles by as much as 50% (Middlemis-Maher et al. 2013). I placed two empty 1-L cups covered with mesh into all other treatments as a control. For the exogenous CORT exposure treatments, I chose nominal CORT concentrations of 62.5 and 125 nM, which will be referred to as low CORT and high CORT treatments, respectively. Previous studies have demonstrated that exposing tadpoles to 125 nM of exogenous CORT can raise whole-body CORT levels by up to 50%, similar to the response to natural stressors (Glennemeier and Denver 2002, Middlemis-Maher et al. 2013). For the treatments receiving MTP, I chose a nominal concentration of 110 μM, which has been shown to reduce whole body CORT levels in tadpoles by over 50% (Glennemeier et al. 2002).

After distributing tadpoles into their treatment tubs, all containers were dosed with their respective treatments. For the 0.5 mg/L carbaryl treatment, I first created a 1:10 dilution of the commercial carbaryl solution in UV-irradiated, aged well water, resulting in a stock concentration of 23,600 mg/L carbaryl. I then added 636 μL of the carbaryl solution to the appropriate experimental unit. Sorption of carbaryl by plastic experimental units is minimal (approx. 0.1%; Bridges 2000). To expose tadpoles to predator cues, I fed each predator ≥300 mg of wood frog tadpole biomass every 24 h. Previous research has found that this level of prey biomass saturates predator cues in the water and maximizes prey responses (Schoeppner and

Relyea 2008). The empty cups in all other treatments were also manipulated at each feeding to replicate the disturbance of feeding. For the CORT treatments, I created a 50 mM corticosterone stock by dissolving corticosterone (≥98.5% HPLC; Sigma-Aldrich, St. Louis, MO; CAS: 50-22-6) in 100% ethanol. To obtain nominal treatment concentrations of 62.5 and 125 nM CORT, I added 37.5 and 75 μL of stock, respectively, to the appropriate experimental units. For the MTP treatments, I created a 6.6 M stock solution by dissolving metyrapone (96%; Sigma-Aldrich, St. Louis, MO; CAS: 54-36-4) in 100% ethanol. To obtain nominal treatment concentrations of 110 μM, I added 500 μL of stock to the appropriate experimental units. Finally, I added ethanol and water to the appropriate treatments as sham exposures to account for solvents. The carbaryl treatments, the CORT treatments, and the MTP treatments were not renewed, and I conducted no water changes over the 72-hr exposure period.

After 72 h of exposure to their respective treatments, tadpoles were transferred into clean 68-L tubs filled with 30 L of aged, UV-irradiated well water (Fig. 1.1). Tadpoles were maintained in these containers for 24 h until the beginning of phase 2 of the experiment. Tadpoles were fed 180 mg of rabbit chow 32 h into the stressor exposure and 180 mg of rabbit chow after being transferred to clean water. After 24 h and immediately prior to the beginning of phase 2, 16 individuals from each phase 1 stressor treatment were immersed in MS-222 for 60 seconds and then individually flash-frozen in vials immersed in liquid nitrogen for later whole-body CORT analysis. I attempted to quantify whole tadpole CORT levels following previously established steroid hormone extraction (Denver 1998) and radioimmunoassay (RIA) methods (Denver 1993). However, even after pooling several samples, I was unable to reliably detect CORT content, which was likely due to the small size of the hatchling tadpoles. Therefore, whole-body CORT levels prior to the experiment were not included in the analysis.

Phase 2: TTD experiments to Assess Induction of increased Pesticide Tolerance

On 6 May, I began phase 2 of the experiment to assess the effects of the phase 1 treatments on pesticide tolerance of the tadpoles (Fig. 1.1). I achieved this by exposing tadpoles from phase 1 to either a no-insecticide control or a lethal carbaryl treatment in time to death (TTD) assays. Time to death assays using concentrations exceeding environmentally relevant concentrations are commonly used to rapidly assess the relative pesticide tolerance of different groups and also provide insight into sublethal effects (Bridges and Semlitsch 2000, Newman 2010, Hua et al. 2015a). For the lethal carbaryl treatment, I chose a nominal carbaryl concentration of 20 mg/L based on previous studies with this same population of wood frogs (Hua et al. 2015b).

Experimental units for this phase were 130-mL plastic cups filled with 50 mL of clean water or carbaryl-treated water. Each experimental unit contained a single tadpole and each of the 16 treatment combinations was replicated 20 times for a total of 320 experimental units. Experimental units were then randomly distributed across two shelves.

I created a carbaryl stock solution by first making a 1:10 dilution of commercial carbaryl solution, as described above, and then adding 8.48 mL of the stock to 10 L of aged, UV-irradiated well water to obtain a nominal concentration of 20 mg/L carbaryl. I then added the clean water and carbaryl-treated water to their respective cups and haphazardly added one tadpole from each phase 1 treatment to their respective phase 2 cups. I conducted water changes every 24 h with a renewal of the pesticide concentration in the carbaryl treatment. I monitored and recorded tadpole mortality every 2 h for the first 12 h and then every 4 h for the next 60 h, terminating the experiment at 72 h. I did not feed the tadpoles during the 72h experimental period, in accordance with standard toxicity tests (ASTM 2008). At the end of the experimental

period, all surviving individuals were euthanized by MS-222 overdose and preserved in 70% ethanol. Animal husbandry and euthanasia procedures were approved by the Purdue University Animal Care and Use Committee (protocol #1701001530).

Pesticide Analysis

I collected one 50-mL water sample from each of the working solutions the day they were created to verify the pesticide concentrations from phase 1 and phase 2 of the experiment. After collection, samples were immediately delivered on ice to Purdue Bindley Bioscience Center (West Lafayette, IN) for analysis. The working solution for the phase 1 sublethal pesticide treatment had a detected concentration of 0.67 mg/L carbaryl. The working solution for the phase 1 sublethal pesticide + MTP treatment had a detected concentration of 0.69 mg/L carbaryl. The working solutions for the phase 2 lethal pesticide treatment had detected concentrations of 27.83, 27.86, and 27.90 mg/L carbaryl after renewal on 6 May, 7 May, and 8 May, respectively. Because the detected carbaryl concentrations were approximately 35% higher than the nominal value in each phase, I will refer to the detected concentrations hereafter.

Statistical Analysis

I performed all statistical analyses using R version 3.5.1 (R Core Team 2018). Survival was high in the phase 2 no-carbaryl control treatments. Because these treatments were only used to monitor background mortality, they were excluded from the analysis. The data did not meet the proportional hazards assumption of the semiparametric Cox's proportional hazard model (tested with cox.zph function). Therefore, I used the nonparametric Gehan-Wilcoxon test (option rho = 1 in the survdiff function) to perform the comparisons (Pyke and Thompson 1986, Hoverman et al. 2010, Wuerthner et al. 2019). Survival analyses were conducted using the

'survival' package in R (Therneau 2015). Survival curves were produced using the 'ggfortify' package in R (Tang and Horikoshi 2016).

First, to determine if exposure to each of the three phase 1 corticosterone synthesis inhibitor (MTP) treatments prevented the larval wood frogs from inducing tolerance to the phase 2 lethal carbaryl challenge, I conducted an overall Gehan-Wilcoxon test comparing survival curves among the three MTP treatments (MTP, MTP + 0.5 mg/L carbaryl, and MTP + predator cue) and the vehicle control. Because the overall test was not significant (Results), no posthoc pairwise comparisons were conducted between the treatments. Both the overall test confirmed that the MTP treatments did not significantly differ from the vehicle control in pesticide tolerance they were not included in subsequent analysis of the stressor treatments.

Second, to assess how exposure to each of the four phase 1 stressor treatments affected the tolerance of larval wood frogs to the phase 2 lethal carbaryl treatment, I conducted an overall Gehan-Wilcoxon test comparing survival curves among the four phase 1 stressor treatments (0.67 mg/L carbaryl, predator-cue, 67.5 nM CORT, 125 nM CORT) and the vehicle control. Because the overall test was significant (Results), I then conducted four pairwise comparisons of the survival curves from the four phase 1 stressor treatments to the survival curve of the tadpoles previously exposed to the ethanol vehicle treatment to directly address the main research questions.

1.4 Results

Survival was high in all sublethal stressor treatments in phase 1 of the experiment (range = 95 – 100%). In phase 2 of the experiment, tadpole survival was high in the no-carbaryl control treatments (range = 90 – 100%), whereas survival was low in the lethal carbaryl treatment at the end of 72 h experimental period (Table 1.1).

In phase 2, I found no significant differences between the survival of the phase 1 corticosterone synthesis inhibitor treatments (MTP) and the control treatment (df = 3, χ^2 = 6, P = 0.11). However, I found a significant overall effect of the phase 1 stressor treatments on the survival curves of wood frog tadpoles (df = 4, χ^2 = 10.8, P = 0.03). Tadpoles previously exposed to low and high CORT in phase 1 displayed increased tolerance to the lethal carbaryl treatment compared to tadpoles from the phase 1 control treatment (low CORT: χ^2 = 4.1, P = 0.043; high CORT: χ^2 = 6.3, P = 0.012; Fig. 1.2A, B), with low CORT increasing median survival time by 50% and high CORT increasing median survival time by 78.6% (Table 1.1). Similarly, tadpoles previously exposed to predator cues in phase 1 also displayed increased tolerance to the lethal carbaryl treatment compared to tadpoles from the phase 1 control treatment (χ^2 = 4.6, P = 0.031; Fig. 1.2C), with a 21.4% increase in median survival time (Table 1.1). Tadpoles previously exposed to the 0.67 mg/L carbaryl treatment in phase 1 did not display increased tolerance to the lethal carbaryl treatment compared to the tadpoles from the phase 1 control treatment (χ^2 = 3.3, P = 0.068; Fig. 1.2D).

1.5 Discussion

As organisms face increasing pressures from anthropogenic pollutants, it is important to understand the different mechanisms by which organisms may cope with exposure to novel stressors in their environment. I explored whether pesticide tolerance in larval wood frogs could be induced by a generalized stress response. I found that prior exposure to a simulated stress event (62.5 and 125 nM of exogenous CORT) induced tolerance to a lethal concentration of carbaryl in larval wood frogs. Similarly, prior exposure to a natural stress event (i.e. predator cues) also increased tolerance to a lethal concentration of carbaryl. In contrast to previous studies, exposure to 0.67 mg/L carbaryl did not significantly increase tolerance to a subsequent

exposure to a lethal carbaryl concentration. I also found that individuals previously exposed to the corticosterone biosynthesis inhibitor metyrapone (MTP), either alone or in combination with a stress cue, did not induce tolerance to a lethal concentration of carbaryl. This provided additional evidence that the tolerance induced by the stress cue was a result of the stress response and not some other response to the cue.

The induction of tolerance to a lethal pesticide concentration following exposure to both predator cues and CORT suggests that induced tolerance to some contaminants, such as carbaryl, can result from a generalized stress response prior to the lethal exposure. One possible mechanism by which a prior stress response may induce tolerance to contaminants is by upregulating one or several metabolic pathways responsible for carbaryl detoxification. For example, glucocorticoids important in the stress response such as CORT have been shown to up-regulate the activity of cytochromes P450 (CYPs), including CYP1A (Konstandi et al. 2014), which plays a key role in metabolizing carbaryl and other environmental contaminants (Tang et al. 2002, Konstandi et al. 2014, Oziolor et al. 2017). Although most studies on the stimulatory effect of glucocorticoids on CYP activity have occurred in mammalian systems (Meredith et al. 2003, Audet-Walsh et al. 2009, Konstandi et al. 2014), the CYP family is highly conserved across vertebrates (Ortiz de Montellano 2005). Thus, the same principles may apply to other taxa and environmental contaminants that are metabolized by the same pathways. For example, a study on induced pesticide tolerance in Gulf killifish (*Fundulus grandis*) found that exposure to carbaryl early in life induced CYP1A activity and increased the ability of the fish to metabolize a lethal carbaryl dose five days later (Oziolor et al. 2017), highlighting the role that CYP1A plays in induced pesticide tolerance in non-mammalian wildlife species. This study suggests that the initiation of a generalized stress response by pesticides or other stressors and the resulting

hormonal cascade may be one detoxification pathway in aquatic organisms. More specifically, increased stress hormone levels prior to lethal pesticide exposure may have indirect preparative effects that bolstered carbaryl tolerance by stimulating metabolic activity (Sapolsky et al. 2000). This highlights the need to consider the role of the stress response in contaminant detoxification and underscores the importance of studying the interplay between metabolic activity and the stress response as a possible mechanism of induced tolerance to other contaminants.

While CYP1A represents one pathway by which a generalized stress response may induce contaminant tolerance in larval wood frogs, there may be multiple physiological responses that play a role in induced carbaryl tolerance. For example, glucocorticoids such as CORT can also reduce the binding time of acetylcholine to neuronal cells, thus mitigating some of the negative effects of acetylcholinesterase enzyme inhibition (Shi et al. 2002). Moreover, wood frog tadpoles have been found to increase the production of acetylcholinesterase (AChE) following exposure to carbaryl (Hua et al. 2013), potentially buffering some of the negative effects of the contaminant. Notably, studies in other systems suggest that following stress, increased glucocorticoid production facilitates increased production of AChE and that the AChE gene contains a glucocorticoid response element that likely facilitates this response (Battaglia and Ogliari 2005, Sailaja et al. 2012, Srinivasan et al. 2013). This suggests that multiple specific mechanisms of contaminant tolerance may be induced by a generalized stress response and that there is typically more than one mechanism responsible for a plastic increase in tolerance. However, the specific mechanisms of tolerance are likely to depend on the characteristics of the contaminant, timescale of exposure, the environmental conditions prior to exposure, and the organism's available mechanisms of response. More focused mechanistic research is warranted to address these questions.

This study found that exposure to both predator cues and exogenous corticosterone induced pesticide tolerance to a similar degree, with no significant difference between the two. This suggests that the CORT treatments largely mimicked the physiological stress response caused by a predator, which has been documented in other studies (Maher et al. 2013). Importantly, because the individuals in the predator cue + MTP treatment did not induce pesticide tolerance, it is likely that the predator stress itself and not some other response to the predator cue-induced tolerance. These findings highlight the crucial role of ecology (e.g. predation) in driving patterns of tolerance or sensitivity to pesticides and suggest that predators can indirectly influence tolerance to pesticides via the stress response. The fact that several studies have shown that predator-induced stress can make carbaryl more deadly to amphibians also merits consideration in light of these findings (Relyea and Mills 2001, Relyea 2003). Though the results presented in this study may seem contradictory to these previous studies, this study differed from these studies in both the timing (predator stress prior to pesticide exposure vs simultaneous exposure) and the duration (3 d vs 10-16 d) of predatory stress exposure. In this study, predator-induced stress prior to pesticide exposure likely upregulated generalized physiological responses to stressors and facilitated the adaptive response to carbaryl, whereas the combined chronic stress from both a predator and pesticide, such as in the studies by Relyea and Mills (2001) and Relyea (2003), was likely harmful (Costantini et al. 2010). Long-term stressors can cause an organism to maintain elevated stress levels that are energetically costly and detrimental to fitness (Calow and Forbes 1998, Sapolsky et al. 2000, Blas et al. 2007). In contrast to a long-term stress response, a short-term stress response can benefit long-term fitness in the face of a stressor by mobilizing energy resources to cope with the stressor and minimize potential damage (Sapolsky et al. 2000). It is important to note that the adaptive value of a short-

term stress response also depends on its magnitude and the frequency with which it is elicited. Although I was unable to quantify CORT levels in the stressor treatments, these tadpoles likely experienced a short-term stress response in the range of what a natural stressor might elicit based on previous studies using similar stressor treatments (Glennemeier and Denver 2002, Maher et al. 2013). While a stress response of this magnitude can be beneficial and was shown to induce pesticide tolerance, stronger or more frequent stress responses may be energetically draining and have a negative impact on pesticide tolerance and other physiological processes (Boonstra 2013, Schoenle et al. 2018). Given the considerable spatial and temporal variation in stressors (Thorp and Bergey 1981, Lewis et al. 2002, Kopp et al. 2006), future studies that manipulate the duration, magnitude, and frequency of stress prior to a lethal pesticide challenge will be valuable in determining potential limitations and constraints of induced pesticide tolerance.

In contrast to previous studies (e.g. Hua et al. 2013b, Jones et al. 2017), prior exposure to a sublethal concentration of carbaryl did not significantly increase pesticide tolerance in this study. However, the sublethal dose of carbaryl did increase median survival time by 35.7% relative to the control treatment. Other studies have found that the degree of tolerance induced by a sublethal dose of pesticide can depend on the dose, exposure time, and developmental stage at exposure (Hua et al. 2013, 2014, Jones et al. 2017). The short-term exposure period (72-h) combined with the sublethal exposure concentration (0.67 mg/L) may not have been enough to induce a strong stress response (Jones et al. 2017). Although sublethal carbaryl exposure did not significantly increase tolerance in this experiment, the other treatments do provide evidence that a generalized stress response is one mechanism of induced tolerance in larval wood frogs. The effects of carbaryl on CORT levels and the mechanisms by which exposure can induce tolerance warrants further investigation.

In conclusion, this study provided evidence that induced tolerance to pesticides is linked to a generalized stress response. Exposure to cues from a predator (i.e. specific, natural stressor) and the stress hormone corticosterone (i.e. simulated general stressor) induced elevated pesticide tolerance in wood frog tadpoles relative to tadpoles with no history of stressor exposure. Because the stress axis is highly conserved across taxa, these results suggest that inducible pesticide tolerance via a generalized stress response may be a ubiquitous mechanism of contaminant tolerance; however, more research is needed in a wider range of taxa before any conclusions about the generalizability of induced tolerance can be reached. Overall, the findings highlight the importance of considering physiological ecology in ecotoxicology as well as identifying mechanisms underlying plastic responses to pesticides. Moreover, this study underscores the crucial role that ecological interactions can have in driving patterns of tolerance and sensitivity to a contaminant.

Table 1.1. Results from the analysis of survival of phase 1 tadpoles during phase 2 exposure to the lethal-carbaryl treatment. Shown for each phase 1 sublethal stressor treatment is the total number of moralities and percent mortality, median survival time, the percent increase in median survival time from the control, and 95% confidence intervals for the median survival estimate.

Phase 1 treatment	***Deaths N (%)***	***Median survival time (hrs)***	***Increase in median survival time from control (%)***	***95% CI***
62.5 nM CORT	15 (75)	42	50	(32 - NA)
125 nM CORT	16 (80)	50	78.6	(36 - 68)
Predator cue	18 (90)	34	21.4	(32 - 44)
0.67 mg/L carbaryl	20 (100)	38	35.7	(32 - 52)
Control	17 (85)	28	NA	(24 - 40)

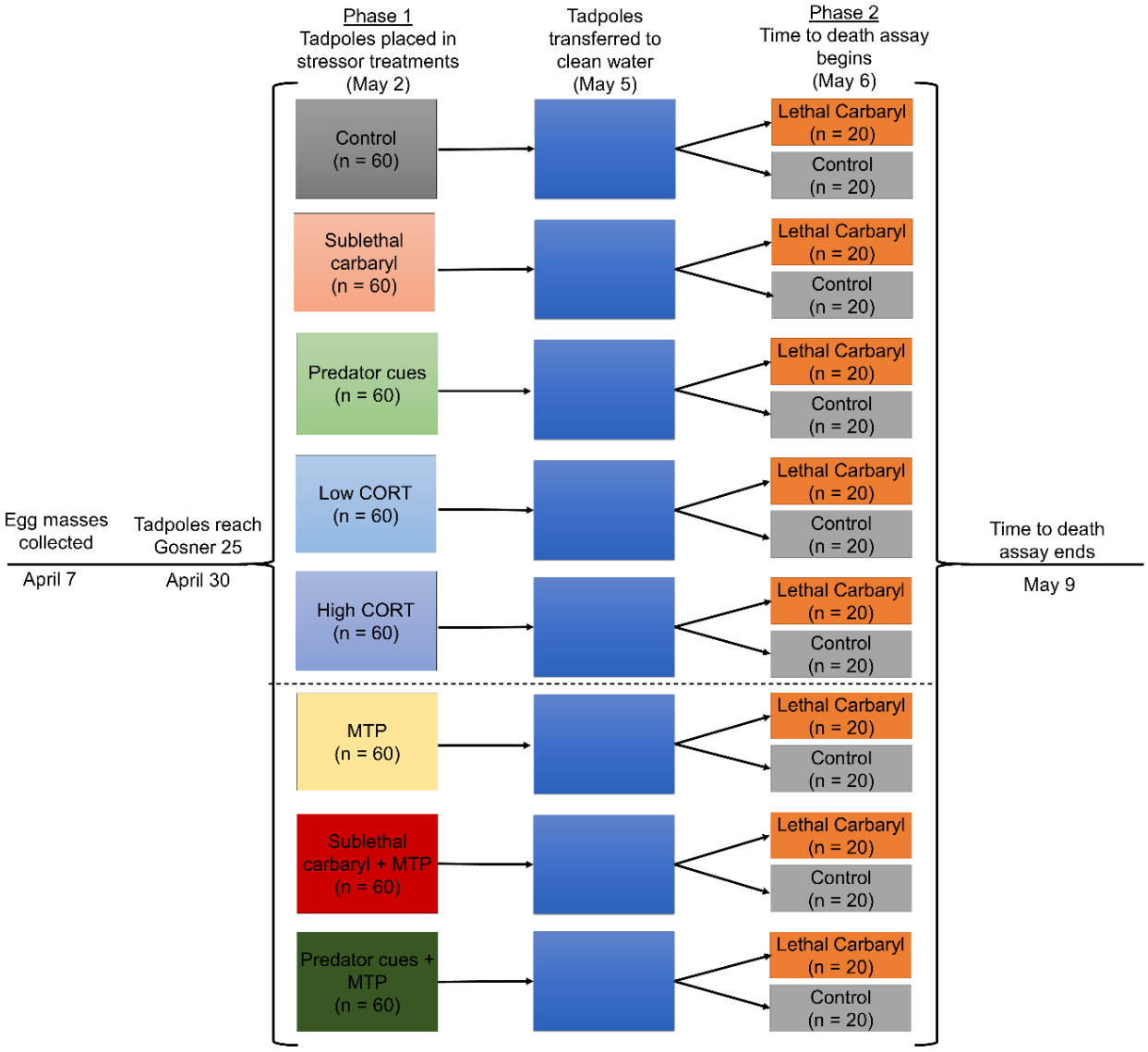

Figure 1.1. Schematic example of the two-phase experimental design. Phase 1 consisted of exposures to one eight pre-treatments (vehicle control , 0.67 mg/L carbaryl, 67.5 nM CORT, 125 nM CORT, predator cue, MTP, 0.69 mg/L carbaryl + MTP, predator cue + MTP). Phase 2 consisted of time to death assays using a lethal concentration of carbaryl (27 mg/L). A dashed line is shown to separate the focal treatments from the MTP treatments.

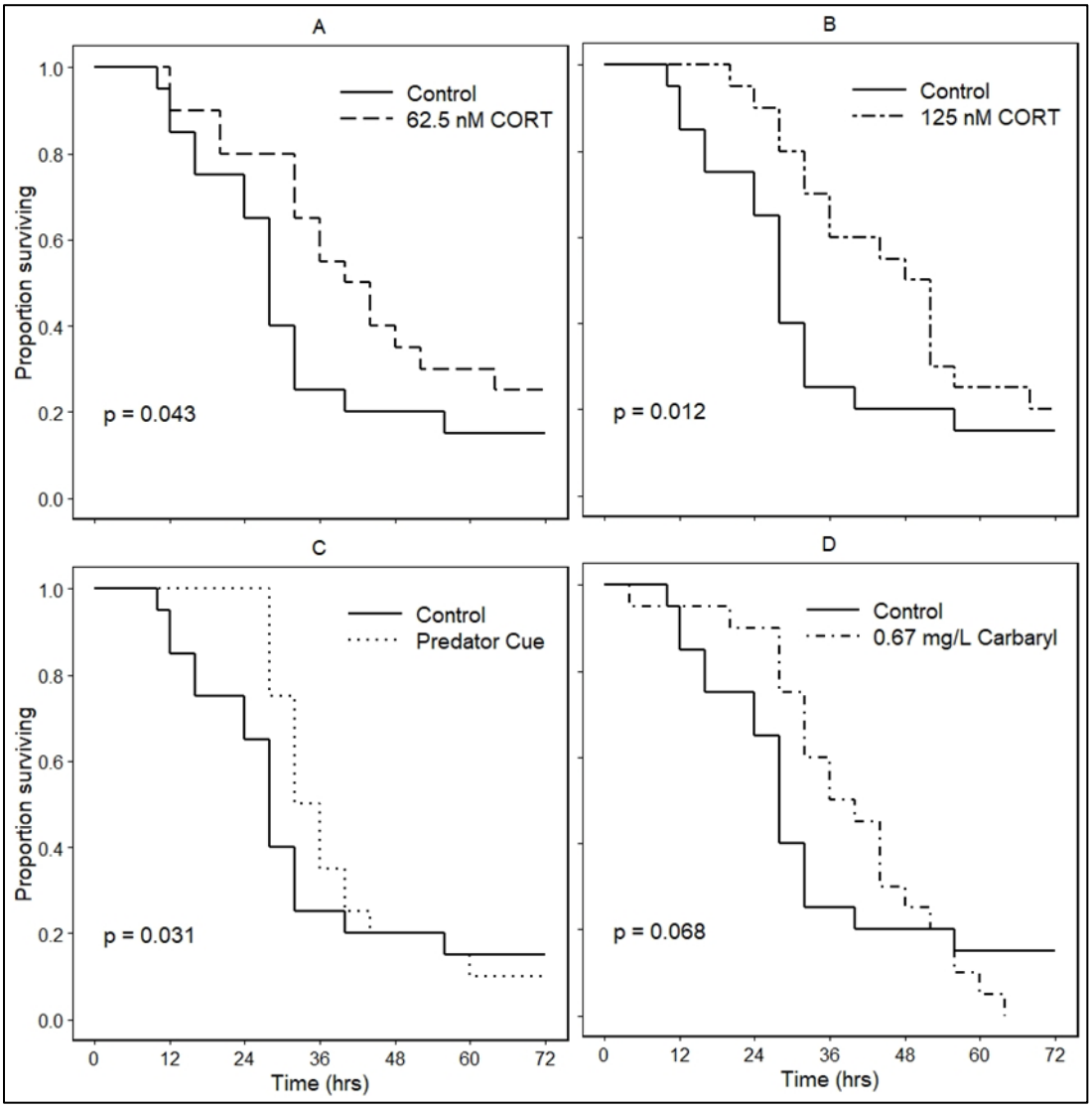

Figure 1.2. Survival curves of wood frog tadpoles exposed to a lethal concentration of carbaryl for 72 h following a previous exposure to sublethal stressor treatments for 72 h. The overall Wilcoxon-Gehan test was significant (χ^2 = 10.8, df = 4, P = 0.03), so subsequent pairwise comparisons were conducted. The survival curve from each of the four sublethal stressor treatments is shown next to the control in four separate panes. P-values for each pairwise comparison are shown beside each curve.

CHAPTER 2. KNOW YOUR ECHINOSTOME: INFECTION OUTCOMES ASSOCIATED WITH COINFECTION BY TWO CRYPTIC TREMATODE SPECIES

2.1 Abstract

The field of disease ecology has placed an increasing emphasis on understanding infection outcomes associated with coinfection by an assemblage of two or more parasites. However, infection outcomes associated with coinfection by functionally and morphologically similar cryptic parasite species remain relatively unexplored. We first conducted observational field surveys in Pennsylvania that revealed the common co-occurrence of cryptic species within the trematode family Echinostomatidae. Based on these findings, I used a larval anuran host (*R. pipiens*) and the two most common cryptic echinostome species, *Echinostoma trivolvis* and *Echinoparyphium* lineage 3, to examine how the timing of host exposure to these functionally similar parasites influences infection outcomes. I found that *Echinostoma* had higher infection success than *Echinoparyphium* in the focal host species in individual exposures, but that simultaneous exposure may increase the infection success of one or both species. I also found that individuals first exposed to *Echinoparyphium* prior to a second parasitic exposure had 22.8% lower infection loads than individuals first exposed to *Echinostoma* prior to a second parasitic exposure, suggest that that exposure timing and order can strongly influence coinfection outcomes, even with functionally similar cryptic parasites. Finally, I show that the encystment of both echinostomes species is biased toward the right kidney over the left kidney, but that exposure order may influence the magnitude of this bias. These findings demonstrate that co-occurring cryptic species likely interact both directly and indirectly and emphasize that species makeup may influence infection outcomes in unexpected ways.

2.2 Introduction

Aquatic environments often contain a diverse community of macroparasite species that make up a significant portion of free-living biomass, play an important role in food webs, and serve as a source of infectious disease (Hechinger and Lafferty 2005, Johnson and McKenzie 2008, Lafferty et al. 2008, Johnson and Hoverman 2012, Johnson et al. 2013, Preston et al. 2013). However, accurate measurement of macroparasite biodiversity in aquatic systems has proven difficult, with modern molecular methods suggesting that many surveys underestimate the true macroparasite biodiversity in aquatic systems (Steinauer et al. 2007, Dobson et al. 2008, Detwiler et al. 2010). This is in large part due to the occurrence of cryptic species, which appear morphologically similar but are genetically distinct (Pfenninger and Schwenk 2007). Because macroparasite species can significantly vary in their ability to cause pathology and interactions between parasite species within a host can alter infection outcomes, studies that examine the outcomes of coinfection with commonly co-occurring cryptic species are valuable (Johnson et al. 2002, Hoverman et al. 2013, Wuerthner et al. 2017).

There is growing evidence that multi-parasite interactions can influence infection outcomes within a host. When a host is challenged by two parasites simultaneously, the coinfection can increase, decrease, or have no effect on the competitive ability of each parasite depending on factors such as competition for resources and cross-reactive immunity (Pedersen and Fenton 2007). Moreover, hosts are likely challenged by different parasite species sequentially due to spatial, diel, and seasonal variations in prevalence and emergence (Fingerut et al. 2003, Studer and Poulin 2012, Marino et al. 2017). Thus, priority effects driven by both exposure timing and order can strongly influence infection outcomes and result in different outcomes compared to simultaneous infection. For example, a study by Hoverman et al. (2013) found that infection of larval amphibians with echinostome trematodes prior to exposure to the

trematode species *Ribeiroia ondatrae* reduced *R. ondatrae* infection success. However, there was no evidence of an interaction between the two parasites with simultaneous exposure. The prevalence of different parasite species is dynamic and variable through space and time, and so understanding the consequences of coinfection on infection outcomes with different parasite species, including cryptic species, will broaden our understanding of disease dynamics in natural populations (Telfer et al. 2008, 2010, Ezenwa et al. 2010, Knowles 2011).

Echinostomes are a diverse and common group of cryptic trematode parasites, with molecular evidence suggesting that over 10 echinostome species exist in the United States (Detwiler et al. 2010). Echinostomes are frequently used as a model parasite in ecological studies, particularly in amphibians due to their ability to cause intensity-dependent pathology (Fried et al. 1997, Holland et al. 2007). Although much of the research on echinostome-driven pathology in amphibians has focused on one species, *Echinostoma trivolvis*, echinostomes can be difficult to identify to species without molecular tools, particularly the cercarial stage that infects amphibians (Kostadinova et al. 2003). Because of this, many ecological studies use field-collected echinostomes with identification limited to family (Echinostomatidae) (e.g., Koprivnikar et al. 2007, Hoverman et al. 2013, Buss and Hua 2018). The frequent co-occurrence of multiple trematode species within an ecosystem suggests that some studies have used two or more cryptic parasite species, with the potential for interactions within the host that may affect infection success and pathology.

Amphibians are an excellent model system for assessing the influence of infection timing and order with cryptic echinostome species on infection outcomes. Amphibians are a second intermediate host for a variety of trematode species and frequently co-occur with echinostomes (Hoverman et al. 2012). Echinostomes and other trematodes typically use freshwater snails as a

first intermediate host. The rediae in the first intermediate host then release free-swimming cercariae into the water column, which enter tadpoles via the cloacal opening and encyst in the kidney as metacercariae (Szuroczki and Richardson 2009). Because there are a considerable number of field and experimental studies of the echinostome-amphibian system, it is of particular interest to explore how echinostomes vary in infection success and how coinfection with two or more co-occurring cryptic species may influence infection outcomes.

Here, I used observational field data of echinostome prevalence across three months in seven ponds to inform laboratory experiments that explore how intraspecific and interspecific interactions between commonly co-occurring echinostome species influence infection outcomes in larval leopard frogs (*Rana pipiens*). Specifically, I examined how the timing of host exposure to two common echinostomes (*Echinostoma trivolvis* and *Echinoparyphium* lineage 3) individually and in combination influence overall infection loads. For three treatments receiving a single exposure to one of the two species or a mixed cohort of the two simultaneously, I predicted that direct interspecific interactions would not strongly influence infection loads and that infection loads would be similar across the treatments. For the treatments receiving two separate exposures to the echinostome species individually or in combination, I predicted that treatments exposed to the two different species sequentially would have lower infection loads than treatments exposed to the same species sequentially due negative intraspecific interactions via priming of the immune system.

2.3 Methods

Field Surveys

As part of a larger project to collect a large quantity of *Helisoma trivolvis* snails infected with only *Echinostoma trivolvis*, we surveyed seven ponds in northwestern Pennsylvania from

May – July 2018. We surveyed each pond once a month and collected *H. trivolvis*, a second intermediate host for echinostomes. We collected snails through unstandardized dipnet sweeps through submerged vegetation with the goal of collecting a minimum of 50 snails per site, keeping only snails >5 mm as snails below this threshold tend to be immature and rarely support infections (Richgels et al. 2013). Because snail prevalence and sampling effort varied by site and month, snail collection numbers also varied (Month: mean [range]; May: 143.7 [32 – 330]; June: 235.7 [125 – 300]; July: 175 [100 – 200]). To screen collected snails for echinostome infection, we isolated them individually in 50-mL tubes filled with 35 mL of UV-irradiated well water and placed them 10 cm under a light source for 1 h to induce cercarial shedding (Szuroczki and Richardson 2009). We identified echinostome-infected snails by placing cercariae on slides under a compound scope following Schell (1985). We then isolated a single cercaria from each echinostome-infected snail in 95% ethanol. Although it is possible that some snails were infected with multiple echinostome species, the overall prevalence of double infections is very low (~2.5%; Sousa 1993, Lafferty et al. 1994). Moreover, experimental work has shown that double infection with echinostomatids is particularly unstable as one species is typically eliminated by the other (Dönges 1972). This makes it unlikely that any one snail was double infected with two echinostome species.

Trematode identification

I extracted the genomic DNA from each trematode sample using a Qiagen DNeasy extraction kit. The internal transcribed spacer region [ITS-1] of ribosomal DNA was amplified by polymerase chain reaction, using forward (BD1, 5’ - GTC GTA ACA AGG TTT CCG TA - 3’, Bowles and McManus 1993) and reverse (4S, 5’ - TCT AGA TGC GTT CGA A(G/A)T GTC GAT G - 3’, Bowles and McManus 1993) primers. The cycling parameters for polymerase chain

reaction were 95°C for 2 min followed by 35 cycles of 94°C for 1 min, 50°C for 45 sec, and 72°C for 1 min, finishing with an elongation at 72°C for 7 min. I then cleaned the PCR products with QIAquick PCR Purification Kit (Qiagen). Sequencing was conducted by the Purdue Genomics Core Facility in the forward and reverse directions using a BigDye terminator kit (Applied Biosystems) and an ABI 3730XL sequencer.

A total of 89 ITS1 sequences were generated. I grouped these 89 sequences in 14 unique haplotypes using DnasSP v6 prior to phylogenetic analysis (Rozas et al. 2017). I aligned one sample representing each of the 14 haplotypes with 23 previously published sequences of echinostomatids (Detwiler et al. 2010) from GenBank automatically using the MUSCLE alignment in the program MEGA version X (Kumar et al. 2018) and then rechecked manually by eye. Phylogenetic analysis was conducted with a Bayesian inference approach (MrBayes 3.2.7a; Ronquist et al., 2012). I used the General Time Reversible plus Invariant sites plus Gamma distributed model of nucleotide substitution for the analysis. Two simultaneous Bayesian runs were conducted (with the default Markov chain Monte Carlo [MCMC] settings), and run for a total of 5.0×10^6 generations per run, sampling trees and parameters every 100 generations and the first 25% of each run was discarded as burn-in (Hua et al. 2016). I rooted the tree with three outgroup taxa. I then used this tree to identify the novel sequences based on Bayesian support values (Fig. 2.1).

Experimental Animal Collection and Husbandry

Based on the field surveys, the two most abundant echinostomatids at the field sites were *Echinostoma trivolvis* and *Echinoparyphium* lineage 3 (see Results). I maintained a subset of the snails infected with *Echinostoma trivolvis* (N = 10) and *Echinoparyphium* lineage 3 (N = 10) in the lab for the experiments. Snails were housed individually in 1-L cups filled with 0.8 L of UV-

irradiated well water and held at 7°C to reduce shedding prior to the start of experiments. One day prior to the start of the experiments, snails were slowly acclimated to 23°C. Snails were fed a mixture of rabbit chow and spirulina powder *ad libitum*. I collected 10 leopard frog (*Rana pipiens*) egg masses from a temporary pond at the Purdue Wildlife Area (PWA) in West Lafayette, IN on 12 April 2018. I distributed the egg masses into 180-L outdoor culturing pools covered with 70% shade cloth and filled with aged well water. After hatching, tadpoles were fed rabbit chow (Purina) *ad libitum* until the start of experiments. Tadpole health was checked daily. Two days prior to the start of experiments, I brought 150 haphazardly chosen leopard frog tadpoles (snout-vent length = 10.92 ± 1.07 SD, stage = 27.5 ± 0.72 SD; Gosner 1960) into the lab to acclimate to indoor conditions (23 °C, 12:12 light cycle).

Experiment 1- Simultaneous exposure

The first experiment examined whether infection loads differed between exposure to one of two echinostome species or a mixed cohort of both parasites. A single tadpole was assigned to each experimental unit. Experimental units were completely randomized across a shelving unit. To reduce the ability of tadpoles to behaviorally avoid the cercariae, the initial parasite exposures took place in 130-mL cups filled with 75 mL UV-irradiated well water. After 12 h, the tadpoles were transferred to a 1-L cup filled with 800 mL of UV-irradiated well water, as cercariae efficacy is highest during the first 8 h after shedding and declines with time (Rohr et al. 2008). Each experimental unit was randomly assigned to one of three treatments: (1) exposure to 50 *Echinostoma* cercariae, (2) exposure to 50 *Echinoparyphium* cercariae, or (3) exposure to 25 *Echinostoma* cercariae and 25 *Echinoparyphium* cercariae simultaneously (N = 10 tadpoles per treatment). To obtain cercariae to add to the experimental units, I individually shed the echinostome-infected snails for 1 h, as described previously, and then homogenized the cercariae

by species in 1-L cups. I collected and counted free-swimming cercariae with a glass pipette under a dissecting scope and transferred the cercariae directly into each experimental unit. While dosing experimental units, I replaced the source parasites every hour with newly shed cercariae so that all cercariae entered experimental units within 2 h of emergence when they are most infective. I checked for tadpole mortality daily for 5 d and fed each tadpole rabbit chow *ad libitum* during the experiment. I ended the experiment after 5 d, as this allows echinostome cercariae to successfully encyst in the kidneys and minimizes time for tadpoles to clear infection (Hoverman et al. 2013). I euthanized (MS-222 overdose) and individually preserved tadpoles in 70% ethanol. Prior to necropsy, I weighed, staged, and measured snout-vent length (SVL) and total length of each tadpole. To quantify echinostome infection load, I first dissected the left and right kidney structures (primary kidney, nephric duct, pronephros; hereafter referred to as left and right kidneys) of each tadpole, placed them between two microscope slides, and counted the total number of metacercariae on each side under a compound microscope (Schotthoefer et al. 2003) I also examined the rest of the body for metacercariae, but all cysts were found in the kidneys. One mortality occurred during this experiment.

Experiment 2- Exposure timing

The second experiment examined how the timing and sequence of exposure of tadpoles to *Echinostoma* and *Echinoparyphium* influence total echinostome infection loads. Each experimental unit was randomly assigned to one of nine factorial treatment combinations of parasite exposure at time 1 (25 *Echinostoma, 25 Echinoparyphium,* or no parasites) and parasite exposure at time 2 (25 *Echinostoma, 25 Echinoparyphium,* or no parasites). These treatments are summarized in Table 2.1. The experimental setup and parasite exposure methods were identical experiment 1. Tadpoles were exposed to parasites on two dates (time 1 = day 0, time 2 = day 3).

At each time, I added either 25 cercariae of the appropriate species or no cercariae to each experimental unit. I checked for tadpole mortality daily and fed each tadpole rabbit chow *ad libitum* during the experiment. The experiment was ended on day 5, 2 d after the second exposure. I euthanized (MS-222 overdose) and individually preserved tadpoles in 70% ethanol. Tadpoles were necropsied as described in experiment 1. Because experiment 1 and experiment 2 occurred simultaneously in the same randomized experimental array, I can draw direct comparisons between the two experiments. Four mortalities occurred during this experiment.

Statistical analyses

Because the field surveys were limited in scope and not standardized, I restricted analysis of the field data to summary statistics and qualitative descriptions to inform the experimental design. The main response variable for the experimental studies was overall infection load (count of metacercariae recovered) because metacercariae of the two focal echinostomes are morphologically difficult to distinguish and molecular identification of so many parasites was unfeasible. I used a negative binomial generalized linear model (nbGLM) for these analyses as the count data were overdispersed. To examine whether infection loads differed between individuals exposed to *Echinostoma* cercariae, *Echinoparyphium* cercariae, or a mixed cohort of both parasites simultaneously (experiment 1; treatments A – C), I conducted a nbGLM with treatment as the main predictor and SVL as a covariate. To examine how the timing and sequence of exposure of tadpoles to *Echinostoma* and *Echinoparyphium* cercariae influence total echinostome infection loads (experiment 2; treatments D – G), I conducted a nbGLM with treatment as the main predictor and SVL as a covariate. For experiment 2, I also conducted a second nbGLM with time 0 treatment and time 1 treatment as the main predictors and SVL as a

covariate to determine if differences in infection loads differed based on the species used for the initial or secondary exposure.

To better determine if intraspecific or interspecific interactions influenced total echinostome infection loads in experiments 1 and 2, I compared the observed metacercarial load of each treatment to an expected value (Hoverman et al. 2013). The expected parasite infection load was calculated by adding together the average number of parasites recovered from treatments exposed to only 25 cercariae at either time 1 or time 2 (treatments H – K, Table 2.1; Fig. 2.3C); this value represents the infection load that would be expected if infection was additive (i.e. no antagonistic or synergistic interactions). This value was then compared to the observed parasite infection load for the treatments exposed to 50 total cercariae (treatments A – G, table 2.1). For example, to determine if interspecific interactions influenced infection load in treatment C (25 *Echinostoma* + 25 *Echinoparyphium* at time 1), I first added the average number of parasites recovered from treatment H (25 *Echinostoma* at time 1) with the average number of parasites recovered from treatment I (25 *Echinoparyphium* at time 1; 9.1 metacercariae + 8.9 metacercariae). I then used a single sample t-test to determine whether the observed infection load differed from the expected infection load for each of the treatments receiving a total of 50 cercariae.

I also conducted a series of Chi-square Goodness of Fit tests to compare the observed distribution of metacercariae recovered from each kidney (left and right) with those expected assuming completely random, equal distribution on the left and right side. I then conducted a series of Chi-square tests of independence to determine whether the distribution of metacercariae recovered from each kidney differed between treatments.

I performed all statistical analyses using R version 3.5.1 (R Core Team 2018). I determined that the metacercarial cyst load count data for treatments A – G was overdispersed and that using a Poisson distribution was inappropriate using the dispersiontest() function in the 'AER' package (dispersion = 2.45, Z = 5.24, P < 0.001; Kleiber and Zeileis 2008). Negative binomial GLMs were conducted using the glm.nb() function in the 'MASS' package (Venables and Ripley 2002). I used the Anova() function in the 'car' package to estimate p-values (Fox and Weisberg 2011). Estimated marginal means were calculated with the 'emmeans' package (Lenth et al. 2019), and Tukey posthoc tests were used to determine where significant differences among the treatments occurred with the cld() function in the 'multcomp' package (Hothorn et al. 2008). I used the t.test() function to compare observed and expected metacercarial loads. Figures were made using 'ggplot2' (Wickham 2016). I did not log-transform SVL, as it was normally distributed, and transformation did not improve normality. I excluded four total individuals (but no more than one in any treatment) from analyses as their kidneys were degraded prior to cyst counting.

2.4 Results

Echinostome identification and prevalence

Using the sequenced ITS region from individual cercariae each obtained from a different *H. trivolvis* snail host, I identified 89 echinostome samples consisting of 14 distinct haplotypes. By comparing these 14 haplotypes to previously published sequences of echinostomatids (Detwiler et al. 2010; Fig 2.1), I identified the samples as belonging to three species: *Echinostoma trivolvis* (haplotypes 10 – 13, N = 37), *Echinostoma revolutum* (haplotype 14, N = 1), and *Echinoparyphium* lineage 3 (haplotypes 1 – 9, N = 51; Table 2.2). *E. trivolvis* and *Ep.* lineage 3 were identified from snails at all seven sites (Fig. 2.2). Both species were identified

individually at 13 out of 21 sampling sessions and both species co-occurred in 7/21 sampling sessions at five different sites. The single *E. revolutum* sample was identified from the June sampling at LOG. *E. trivolvis* prevalence in snail hosts by month and site ranged from 0 – 6% and *Ep.* lineage 3 prevalence ranged from 0 – 12.5%. The field surveys provided substantial evidence that *E. trivolvis* and *Ep.* lineage 3 commonly co-occur in this study region, thus I selected them as the focal parasites for the experimental studies.

Experiment 1- Simultaneous exposure

For the hosts in the three treatments that experienced a single exposure at time 1 to one of two echinostomatid species or a mixed cohort of both parasites (A – C, Table 2.2), I found evidence that infection loads significantly differed between the three treatments ($\chi^2 = 6.4$, df = 2, P = 0.041; Fig. 2.3A). The mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* was 58.2% higher than the mean metacercarial load of individuals exposed to *Echinoparyphium* alone (Z = 2.353, P = 0.048). Although individuals exposed to only *Echinostoma* tended to have higher infection loads than individuals exposed only to *Echinoparyphium*, this difference was not statistically significant (Z = -1.969, P = 0.120). Mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* was similar to the mean metacercarial load of individuals exposed to only *Echinostoma* (Z = -0.313, P = 0.947). I did not find that infection loads in any treatment differed significantly from the expected value calculated from treatments H – K ($t \leq 2.0$, df = 9, $P \geq 0.076$; Fig. 2.3A).

Experiment 2- Exposure timing

For the hosts in the four treatments that experienced two parasitic exposures to either *Echinostoma* or *Echinoparyphium* at time 1 and time 2 (D – G, Table 2.2), I found evidence that

mean metacercarial load was significantly different between treatments (χ^2 = 12.8, df = 1, P = 0.005; Fig. 2.3B). Mean metacercarial load of individuals exposed to *Echinoparyphium* at time 1 and *Echinostoma* at time 2 (treatment G) was ~36.8% lower than either treatment with exposure to *Echinostoma* at time 1 (treatment D: Z = 3.029, P = .013; treatment F: Z = 3.121, P = 0.01). The mean metacercarial load of individuals exposed to *Echinoparyphium* at time 1 and *Echinoparyphium* at time 2 (treatment E) did not significantly differ from the other three treatments (Fig. 2.3B). I also found evidence that mean metacercarial load was significantly influenced by the parasite species used at exposure at time 1 (χ^2 = 6.4, df = 1, P = 0.012). Following both exposures, mean metacercarial load of individuals exposed to *Echinostoma* at time 1 (combined treatments D and F) was 30% higher than mean metacercarial load of individuals exposed to *Echinoparyphium* at time 1 (combined treatments E and G), regardless of time 2 treatment (χ^2 = 3.3, df = 1, P = 0.07). When comparing expected infection loads with observed infection loads by treatment, mean metacercarial load of individuals in treatment G (Exposure to *Echinoparyphium* at time 1, exposure to *Echinostoma* at time 2) was 32% lower than the expected value (t = -3.98, df = 9, P = 0.003; Fig. 2.3B). Observed infection loads did not differ significantly from the expected value in the other three treatments (all P > 0.05; Fig. 2.3B). I found no significant differences in mean metacercarial load between any of the treatments receiving 25 cercariae at either time 1 or time 2 (treatments H – K) that were used to calculate expected infection values (χ^2 = 2.7, df = 3, P = 0.436).

Kidney encystment bias

Of the 4375 cercariae used across all 11 treatments, 1816 successfully encysted (41.5%). Of those that successfully encysted, 715 encysted in the left kidney (39.4%) and 1101 encysted in the right kidney (60.6%), and a Chi-Square Goodness of Fit test found that the bias toward the

right kidney was statistically significant ($\chi^2 = 82.0$, df = 1, $P < 0.001$). The bias of metacercariae toward the right kidney was consistent within all seven treatments that received 50 cercariae ($P < 0.05$; Fig. 2.4).

When comparing the distribution of metacercariae in the kidneys between treatment groups with a Chi-Square test of independence (e.g., distribution of metacercariae in one treatment vs another treatment), I found no significant differences between the three treatments receiving 50 cercariae simultaneously ($\chi^2 = 1.89$, df = 2, $P = 0.388$) nor did I find significant differences between the four treatments that received 25 cercariae at time 1 and 25 cercariae at time 2 ($\chi^2 = 4.62$, df = 3, $P = 0.202$); however, there was a marginally significant difference in the distribution of metacercariae between individuals exposed to *Echinostoma* at time 1 (D and F combined) and individuals exposed to *Echinoparyphium* at time 1 (E and G combined) ($\chi^2 = 3.62$, df = 1, $P = 0.057$); individuals that received *Echinoparyphium* at time 1 tended to have a higher proportion of cysts in the right kidney (63.4%) than individuals that received *Echinostoma* at time 1 (56.5%).

2.5 Discussion

In natural systems, hosts are typically challenged by a wide array of co-occurring parasites (Rynkiewicz et al. 2015). Accordingly, disease ecologists have shifted focus from studies of single parasite infection outcomes to an assemblage of two or more parasites to better understand the influence of co-infection in disease outcomes, within-host parasite communities, and community disease dynamics (Telfer et al. 2010, Hoverman et al. 2013, Ezenwa 2016, Wuerthner et al. 2017). While this research has demonstrated that coinfection, even with two functionally different parasites, can have profound impacts on infection outcomes, infection outcomes resulting from coinfection with functionally and morphologically similar cryptic

species have received little attention (Miura et al. 2005, Detwiler et al. 2010, 2012). Using molecular methods, I found that two echinostome species, *Echinostoma trivolvis* and *Echinoparyphium* lineage 3, commonly co-occur in aquatic ecosystems. Using this qualitative field survey to inform the experimental design, I demonstrated that both simultaneous and staggered exposure to these two cryptic echinostome species can alter infection outcomes in a larval amphibian host.

In treatments that experienced a single exposure to one of two echinostome species or a mixed cohort of both parasites (A – C; Table 2.1), I found that mean metacercarial load of individuals exposed to a mixed cohort of *Echinostoma* and *Echinoparyphium* was 58.2% higher than the mean metacercarial load of individuals exposed to only *Echinoparyphium* but similar to the mean metacercarial load of individuals exposed to only *Echinostoma.* Individuals exposed to only *Echinostoma* had 48.5% higher infection loads than individuals exposed only to *Echinoparyphium*, but this difference was not statistically significant. These results suggest that co-occurring echinostome species can be highly variable in their ability to successfully infect larval amphibians, but that simultaneous infection with multiple species may result in infection loads equal to or higher than either species individually.

One potential mechanism for this observation is that in coinfection, one parasite facilitated increased infection success in the other. Because of the temporal proximity of exposure to both parasites, it is unlikely that an immune response played a significant role in facilitation, as there is typically a time delay to mount an adaptive immune response (Marshall et al. 2018). Instead, infection by the more successful parasite may have increased the infection success of the other parasite by enhancing its ability to find entry into the host. Studies have documented that different echinostome species are better at detecting cues from specific second

intermediate hosts (Anderson and Fried 1987, McCarthy and Kanev 1990, Haas et al. 1995, Maldonado et al. 2001), and that trematode cercariae display a preference for more susceptible host species (Sears et al. 2012). While it has been confirmed that *E. trivolvis* cysts recovered from the focal host used in this study, *R. pipiens*, are viable and infective to definitive hosts, no such information exists for *Ep.* lineage 3 (Fried et al. 1997). Thus, if *R. pipiens* is a suboptimal host for *Ep.* lineage 3, there should be limited selection on the parasite to detect and successfully encyst in this host. However, if the cellular damage caused by *E. trivolvis* encystment increases the concentration of chemical cues released from the cloaca (e.g., amino acids; Haas et al. 2000), this may increase *Ep.* lineage 3 infection success.

Alternatively, hosts exposed to only *Ep.* lineage 3 at time 1 may have had lower infection loads due to density-dependent regulation resulting from intraspecific interference or a stronger behavioral response by the host (Ebert et al. 2000, Karvonen et al. 2003, Poulin 2010). There may be an evolved mechanism to avoid penetration of previously infected hosts, although this has mostly been explored with the miracidia stage than the highly abundant cercaria stage (Haas et al. 2000, Allan et al. 2009, Vannatta et al. 2020). Because the metacercariae of the two species used are morphologically indistinct, it remains uncertain how coinfection influenced the success of each species individually; however, the patterns in this experiment suggest that infection success can differ between echinostome species but that simultaneous coinfection may increase the infection success of one or both species.

In treatments that experienced parasitic exposure to either *Echinostoma* or *Echinoparyphium* at two separate time points, I found that the mean metacercarial load of individuals exposed to *Echinoparyphium* at time 1 and *Echinostoma* at time 2 was 36.8% lower than either treatment with exposure to *Echinostoma* at time 1. I also found that individuals

exposed to *Echinoparyphium* at time 1 had a 22.8% lower infection load relative to individuals exposed to *Echinostoma* at time 1. Together, these results demonstrate that priority effects can influence the overall infection success of two co-occurring cryptic echinostome species, but that the interaction may be asymmetric and dependent on exposure order. These results are unlikely to be explained by the energetic demands of the previously encysted parasites, as metacercariae have low resource demands (Smyth and Halton 1983). Instead, the finding that initial exposure to *Echinoparyphium* resulted in overall lower infection loads than initial exposure to *Echinostoma* could indicate this that either 1) *Echinoparyphium* exposure induces a strong immune response that confers cross-immunity to a later echinostome infection or 2) *Echinostoma* exposure results in helminth-induced immunosuppression that prevents cross-immunity to a later echinostome exposure from occurring. Helminth-induced immunosuppression is a well-documented strategy used by trematodes to increase their survival in the host (Maizels et al. 2009, Taylor et al. 2012), but it is unclear if this occurs in the metacercarial stage that infects amphibians. There is, however, evidence to suggest that early exposure to trematode cercariae can heighten resistance to a later parasite challenge (Hoverman et al. 2013, Wuerthner et al. 2017, Koprivnikar et al. 2019). The fact that mean infection load of individuals exposed to *Echinoparyphium* at time 1 and *Echinostoma* at time 2 was significantly lower than the expected value supports the cross-immunity hypothesis and suggests that the *E. trivolvis* at time 2 had an infection success lower than what would be expected if infection was additive (i.e., antagonism). Collectively, these results suggest that exposure timing and order can strongly influence coinfection outcomes, even with functionally similar cryptic parasites; however, more research is needed to reveal the mechanism underlying these priority effects.

I found evidence that encystment of echinostome cercariae was biased toward the right kidney structure (60.6% of total cysts), suggesting that echinostomes preferentially encyst in the right kidney of larval anurans (Thiemann and Wassersug 2000, Holland et al. 2007, Orlofske et al. 2009). This result is consistent with previous literature and has been viewed as a possible co-evolved relationship between host and parasite that decreases the risk of renal failure increasing the survival of both players (Thiemann and Wassersug 2000). In light of this, it is interesting that I found that individuals exposed to *Echinoparyphium* at time 1 tended to have a higher proportion of cysts in the right kidney (63.4%) than individuals exposed to *Echinostoma* at time 1 (56.5%), although the difference was not statistically significant ($\chi^2 = 3.62$, $P = 0.057$). When considered in light of the finding that individuals exposed to *Echinoparyphium* at time 1 had significantly lower infection loads, a possible explanation is that as competition for resources (e.g., space) in the right kidney structure increased, more cercariae began to encyst in the left kidney structure. Although I observed minimal mortality in the experiment because of relatively low infection loads relative to those that have been found in natural systems (>1,000 metacercariae; Johnson and McKenzie 2008), a shift in infection bias toward a more even distribution between the kidney structures due to interactions between coinfecting echinostome species could lead to increased pathology and mortality.

An obvious limitation of this study is that in coinfected animals, I was unable to differentiate between the cysts of two echinostome species. Although several studies of amphibian trematode infections have used fluorescing dye to label cercariae used for exposures on different days, the only successful reports of this method appear to focus on *Ribeiroia ondatrae*, which are much larger than echinostomes as cercariae and encyst in the limb bud rather than internally (LaFonte and Johnson 2013, Hoverman et al. 2013, LaFonte et al. 2015,

Koprivnikar et al. 2019). The validation of this method with echinostomes would allow for a more fine-grained understanding of how coinfection timing influences infection outcomes for multiple cryptic species separately. Another limitation is that because the field surveys were unstandardized and limited in scope, few conclusions can be drawn about how the prevalence of each species changes through the year in natural systems. For example, a temporal gap in peak abundance of *E. trivolvis* and *Ep.* lineage 3 could lead to predictably staggered infections similar to those explored experimentally here. Alternatively, spatial separation of snails infected with different echinostomes within a pond could lead to temporal gaps in exposure based on host habitat choices. Further standardized surveys will be needed to determine if this is the case. Finally, because echinostome infection loads can significantly vary in natural situations, the narrow range of exposures likely does not capture the range of outcomes, such as increased pathology or mortality, that might occur with echinostome coinfections. Future studies should explore how coinfection with cryptic echinostomes at a range of exposure loads alters disease outcomes and the success of each parasite.

As the field of disease ecology continues to grow, clarifying the dynamics of within-host parasite interactions will become increasingly important (Pedersen and Fenton 2007, Johnson et al. 2015). This is especially important for within-host interactions between cryptic parasite species, which have remained relatively unexplored. As molecular studies continue to reveal genetic distinctions between morphologically similar species, it is important to explore whether there are non-additive interactions between cryptic macroparasite species or whether the effects of parasite exposure can be generalized by morphotype. The study demonstrates that echinostome infection success in larval anurans can differ significantly based on the species makeup, density, and timing of exposure. I also found evidence for priority effects based on

exposure order; individuals exposed to *Echinoparyphium* at time 1 tended to have lower final infection loads than individuals exposed to *E. trivolvis* at time 1. This finding adds to the existing literature demonstrating priority effects during coinfection and emphasizes that priority effects can occur even between functionally similar cryptic species (Hoverman et al. 2013, Devevey et al. 2015, Wuerthner et al. 2017). Given these findings, I recommend that workers using field-collected echinostomes as a model parasite for disease studies use molecular methods to confirm which species will be used. While the cost of sequencing can be prohibitive, techniques such as qPCR or PCR with species-specific primers (e.g., Fujino et al. 1997) may provide a more affordable way to rapidly identify the presence or absence of DNA from a specific species. Studies should continue to focus on how cryptic parasite diversity in natural systems influences disease outcomes, and how these functionally similar organisms interact and compete for within-host resources.

Table 2.1. Summary of the 12 experimental treatments used in experiments 1 and 2 (N = 10 per treatment). In experiment 1, *Rana pipiens* tadpoles experienced a single exposure to 50 *Echinostoma trivolvis*, 50 *Echinoparyphium* lineage 3, or 25 cercariae of each species at time 1. In experiment 2, *R pipiens* tadpoles were exposed to a factorial combination of 25 *E. trivolvis*, 25 *Ep.* lin. 3, or no parasites. Time 1 = day 0, time 2 = day 3.

Experiment	Treatment	Time 1	Time 2	Total
1	A	50 *E. trivolvis*	No parasites	50
	B	50 *Echinoparyphium*	No parasites	50
	C	25 *E. trivolvis* + 25 *Echinoparyphium*	No parasites	50
2	D	25 *E. trivolvis*	25 *E. trivolvis*	50
	E	25 *Echinoparyphium*	25 *Echinoparyphium*	50
	F	25 *E. trivolvis*	25 *Echinoparyphium*	50
	G	25 *Echinoparyphium*	25 *E. trivolvis*	50
	H	25 *E. trivolvis*	No parasites	25
	I	25 *Echinoparyphium*	No parasites	25
	J	No parasites	25 *E. trivolvis*	25
	K	No parasites	25 *Echinoparyphium*	25
	L	No parasites	No parasites	0

Table 2.2. Haplotype frequencies of the 89 ITS1 sequences obtained from echinostome cercariae collected during field surveys for this study.

Haplotype	Species	N (% of total)
1	*Echinoparyphium* lineage 3	43 (48.3%)
2	*Echinoparyphium* lineage 3	1 (1.1%)
3	*Echinoparyphium* lineage 3	1 (1.1%)
4	*Echinoparyphium* lineage 3	1 (1.1%)
5	*Echinoparyphium* lineage 3	1 (1.1%)
6	*Echinoparyphium* lineage 3	1 (1.1%)
7	*Echinoparyphium* lineage 3	1 (1.1%)
8	*Echinoparyphium* lineage 3	1 (1.1%)
9	*Echinoparyphium* lineage 3	1 (1.1%)
10	*Echinostoma trivolvis*	21 (23.6%)
11	*Echinostoma trivolvis*	14 (15.7%)
12	*Echinostoma trivolvis*	1 (1.1%)
13	*Echinostoma trivolvis*	1 (1.1%)
14	*Echinostoma revolutum*	1 (1.1%)

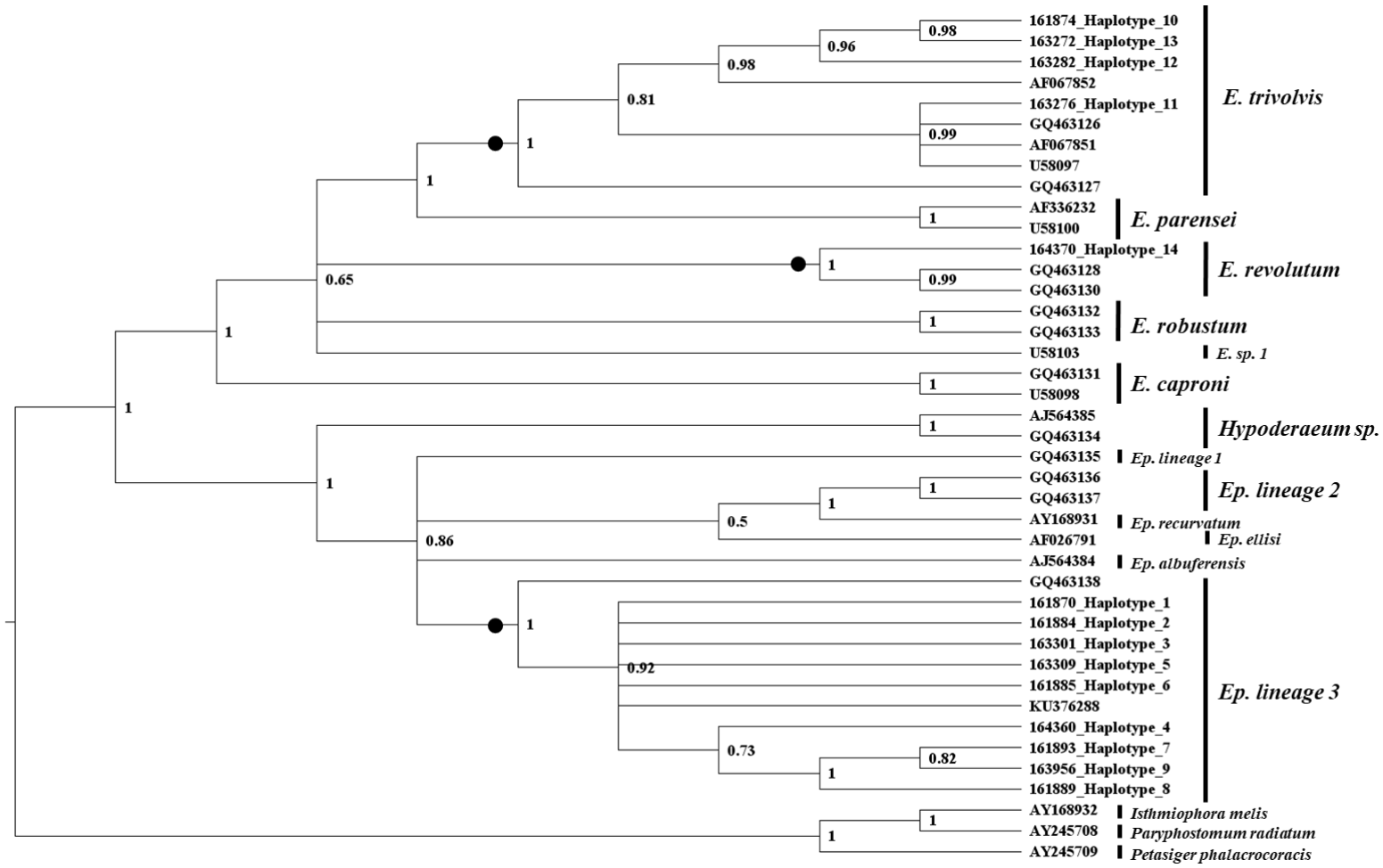

Figure 2.1. Phylogenetic estimate of relationships within Echinostomatidae based on the ITS1 gene inferred from Bayesian support values and rooted with three outgroup species. Support values are shown near the nodes. Nodes supported by ≥ 95% posterior probability are considered highly supported. The circles denote the species or lineages that were detected in the samples.

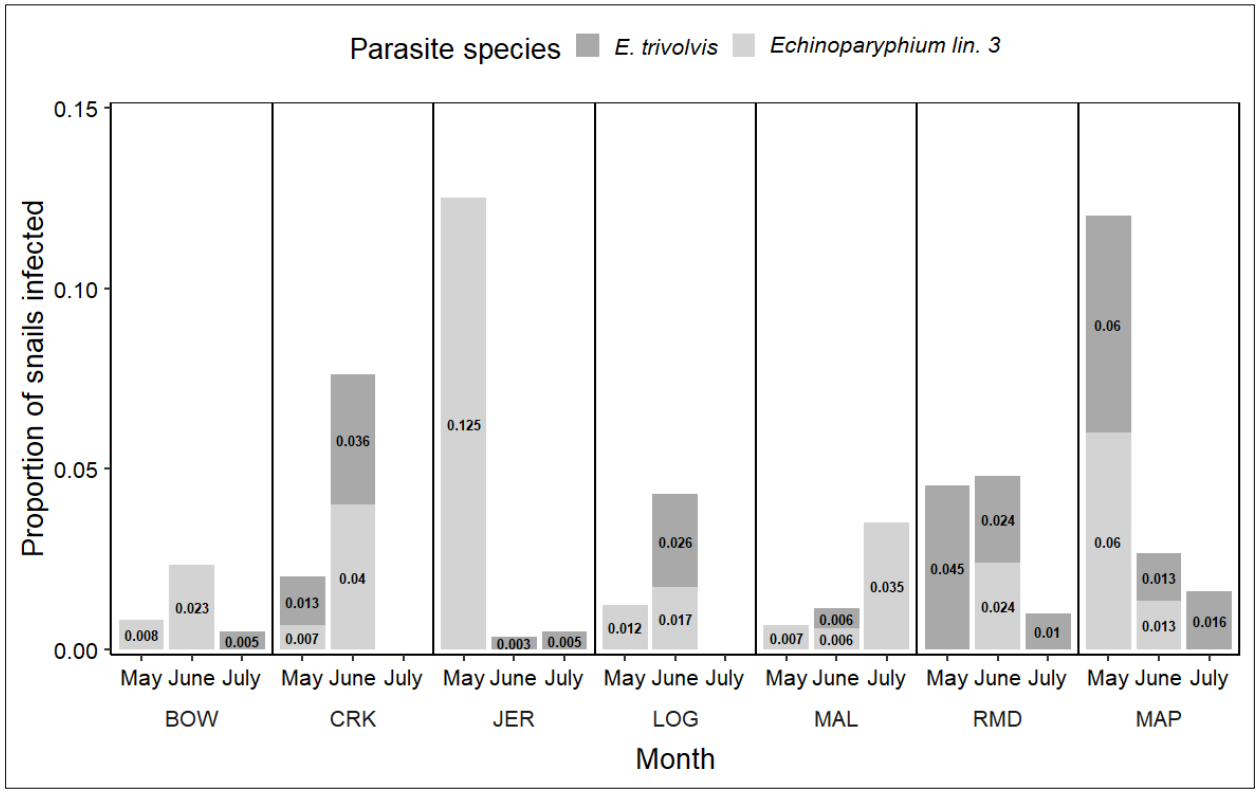

Figure 2.2. The prevalence of *Echinoparyphium* lineage 3 (*Echinoparyphium* lin. 3; light gray bars) and *Echinostoma trivolvis* (*E. trivolvis*; dark gray bars) in seven ponds in northwest Pennsylvania over a three-month survey in 2018. Each bar depicts the proportion of trematode-infected *Helisoma trivolvis* snails, separated by trematode species.

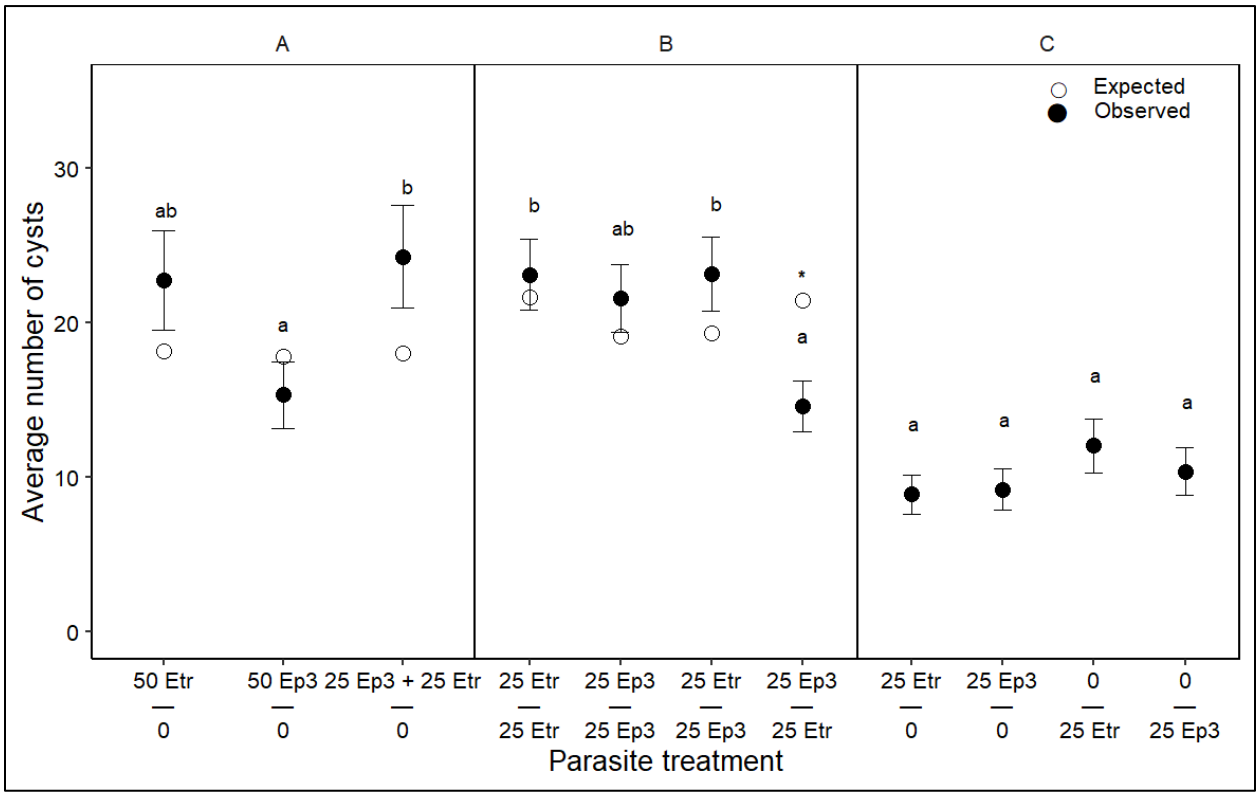

Figure 2.3. Parasite infection loads (estimated marginal mean number of metacercariae recovered ± SE) in *R. pipiens* tadpoles after exposure at one or two time points to different combinations of two echinostome species. For each treatment, the time 1 exposure is shown above the time 2 exposure and separated by '—'. Panel A shows the treatments receiving 50 cercariae at once at time 1; Panel B shows the treatments receiving 25 cercariae at time 1 and 25 cercariae at time 2; Panel C shows the treatments receiving 25 cercariae at either time 1 or time 2. Within each panel, treatments sharing lower case letters are not significantly different from each other ($P > 0.05$). For each treatment in A and B, the expected total infection load calculated from mean infection loads in panel C is shown as an open circle. An asterisk denotes a significant difference between the expected and observed value ($P < 0.05$). Ep3 represents *Echinoparyphium* lineage 3 and Etr represents *Echinostoma trivolvis*.

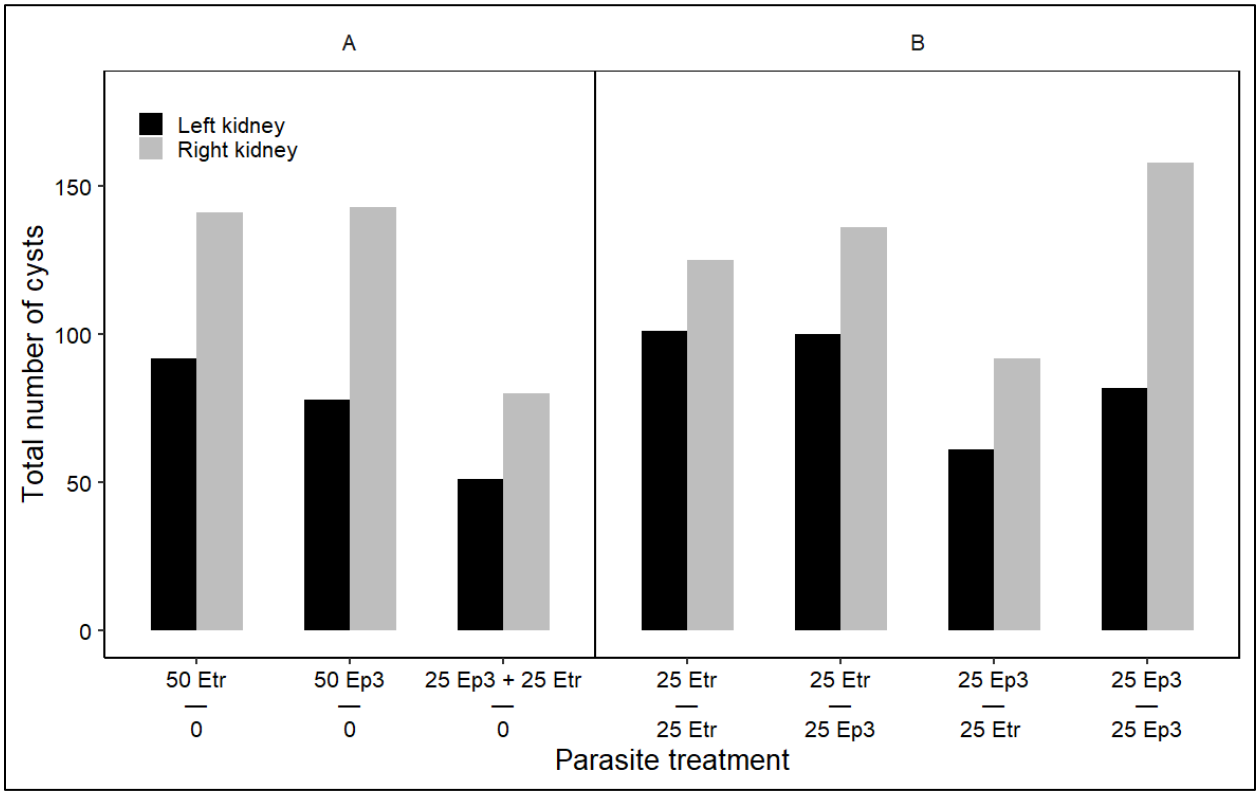

Figure 2.4. Number of cysts in the left and right kidney for treatments receiving 50 total echinostome cercariae. Panel A shows the treatments receiving 50 cercariae at once at time 1 and panel B shows the treatments receiving 25 cercariae at time 1 and 25 cercariae at time 2. Abbreviations are described in Fig. 2.3.

CHAPTER 3. POPULATION-LEVEL VARIATION IN PARASITE SUSCEPTIBILITY NOT INFLUENCED BY PESTICIDE EXPOSURE IN LARVAL WOOD FROGS (*RANA SYLVATICA*)

3.1 Abstract

While it is well-documented that responses to pesticides and parasites vary among amphibian species, there is considerably less literature exploring this variation at the population-level. Moreover, it is unclear how pesticide exposure might differentially influence parasite susceptibility among different populations. Using eight wood frog populations (*Rana sylvatica*), I explored how exposure to the insecticide carbaryl differentially influences infection outcomes from two functionally different parasites, echinostomes and ranavirus. I did this by exposing tadpoles to the pesticide carbaryl (1 mg/L) or a no-pesticide control for 5 d, followed by exposure to one of two of the focal parasites. I found that there was significant population-level variation in trematode and ranavirus infection outcomes. Moreover, I found that the variation in trematode susceptibility was related to a population's proximity to agriculture, with tadpoles from populations located close to agriculture being more susceptible to trematode infections compared to individuals from populations farther from agriculture. However, I found no significant effects of carbaryl exposure on disease outcomes. This study provides evidence that populations can vary significantly in susceptibility to pathogens, but that pesticide exposure may not always increase parasite susceptibility. Further research is needed to determine how environmental variation among populations impact susceptibility to parasites, and if these patterns of susceptibility are consistent across space and time.

3.2 Introduction

Pathogens and parasites, though often inconspicuous, play an important role in many ecosystem processes, including nutrient cycling, community structuring, and host population dynamics (De Castro and Bolker 2005, Wood et al. 2007, Wood and Johnson 2015, Mischler et al. 2016). Indeed, parasites make up a considerable proportion of global biodiversity and can comprise a substantial amount of biomass in healthy ecosystems (Kuris et al. 2008, Dobson et al. 2008). Despite the importance of parasites to natural systems, however, infectious diseases have become an increasing threat to the biodiversity of plants and wildlife in recent decades (Jones et al. 2008). Though a variety of biotic and abiotic factors influence disease outbreaks and epidemics, it has become increasingly clear that human-induced alterations to the environment, such as climate change, pollution, and habitat destruction, are driving many of these disease events (Daszak et al. 2001).

Pesticides have played an important role in disease management, pest control, and agricultural productivity since the beginning of their mass production over 70 years ago. However, the widespread use of these chemicals can have ramifications for nontarget wildlife species that exist near agricultural operations (Pimentel 2005). Although pesticide applications are typically targeted, they can be distributed across the landscape through aerial drift, runoff, and leaching, potentially exposing wildlife species (Stone et al. 2014, Toccalino et al. 2014). There is considerable evidence that pesticide exposure can negatively influence growth, development, behavior, immune function, and survival in a variety of taxa (Egea-Serrano et al. 2012, Gill et al. 2012, Köhler and Triebskorn 2013). Moreover, pesticide-mediated effects on immune function have sparked research exploring the broader implications for disease dynamics. For instance, pesticide exposure can result in increased infection loads, reduced tolerance to

infection, and greater pathology associated with parasitic exposures (Kiesecker 2002, Christin et al. 2003). However, the specific effects of pesticide exposure on disease resistance and immune function are influenced by exposure duration, pesticide concentration, host life stage, and species (Buck et al. 2015, Pochini and Hoverman 2017b). Therefore, it is important to consider the potential consequences of pesticide exposure on host-parasite interactions at different levels of biological organization.

It is well-documented that different species vary in their sensitivity and responses to pesticides (Bridges and Semlitsch 2000, Hammond et al. 2012). Moreover, it is becoming increasingly clear that there can be significant variation in pesticide sensitivity among populations of the same species as a result of both a response to environmental pesticide contamination (i.e. evolved tolerance) and random variation in trait values between populations (Bridges and Semlitsch 2000, Brausch and Smith 2009, Cothran et al. 2013, Hua et al. 2013, Bendis and Relyea 2014). Therefore, although pesticide exposure can be costly, differences in pesticide sensitivity among populations may influence interspecific interactions like host-parasite relationships. For example, populations of *Daphnia magna* selected for higher tolerance to the insecticide carbaryl were more susceptible to parasitic infections than control populations (Jansen et al. 2011), highlighting potential costs and tradeoffs associated with genetic adaptation. Alternatively, disease susceptibility in populations with higher baseline pesticide tolerance may be less impacted by pesticide exposure if increased pesticide tolerance reduces the cost of pesticide exposure.

Amphibians present an excellent system to explore how pesticide exposure differentially influences disease outcomes among different populations because their aquatic habitats often experience pesticide contamination and disease is a major driver of declines globally (Kiesecker

2002, Daszak et al. 2003). Infectious disease outcomes in the context of pesticide exposure have been extensively studied in amphibians, particularly with two common infectious agents, echinostomes and ranaviruses (Forson and Storfer 2006, Rohr et al. 2008, Pochini and Hoverman 2017a, 2017b). Echinostomes (family: Echinostomatidae) are a diverse group of generalist trematode parasites. Echinostomes have a complex life cycle, using larval amphibians, snails, and fish as secondary intermediate hosts. Amphibians are infected by the free-swimming cercarial life stage, which encysts in the kidneys. Pathology is dose-dependent with edema, reduced growth, and mortality occurring at high parasite loads (Johnson and McKenzie 2008). Ranaviruses are a common pathogen in North American amphibian communities and have been implicated in die-off events of larvae and recently metamorphosed individuals (Miller et al. 2011, Duffus et al. 2015). Ranaviruses cause cell death in the liver, kidney, and spleen, often leading to mortality in 7-10 days (Gray et al. 2009). Importantly, pesticide exposure is known to increase susceptibility to both pathogens (Rohr et al. 2008, Pochini and Hoverman 2017b). However, the degree to which pesticide exposure differentially influences disease outcomes across populations of the same species remains unclear.

My objective was to examine how exposure to the insecticide carbaryl differentially influences susceptibility to ranavirus and echinostomes among larvae from different wood frog (*Rana sylvatica*) populations. I chose larval wood frogs as the focal species because populations can vary in their sensitivity to pesticides and pathogens, and their variation in pesticide sensitivity has been found to correlate with a population's distance to agriculture (Cothran et al. 2013, Hua et al. 2013, 2015b, 2017). To achieve this objective, I first exposed wood frog larvae from eight populations to one of two treatments: a sublethal carbaryl concentration (1 mg/L) or a pesticide-free control. Then, I conducted experimental parasite exposures to either echinostome

trematodes or ranavirus to determine how pesticide exposure influenced infection loads, infection prevalence, and survival in each population. If pesticide exposure has an immediate immunosuppressive effect on larval wood frogs, I predicted higher trematode and ranavirus infection loads, higher ranavirus infection prevalence, and lower survival after ranavirus exposure for treatments previously exposed to carbaryl compared to those not exposed. Based on previous work in this system, I also expected population-level variation in how pesticide exposure influences disease outcomes. Specifically, I predicted that the infection outcomes of the populations close to agriculture would be less influenced by pesticide exposure than populations far from agriculture because these populations are generally less sensitive to pesticides.

3.3 Methods

Pesticide background

I used the carbamate insecticide carbaryl (98.1% pure; ChemService, West Chester, PA, USA) for this study. First registered in 1959, carbaryl is a reversible acetylcholinesterase inhibitor that was heavily used for agricultural pest control (Čolović et al. 2013). While its usage in agriculture has declined, carbaryl is still commonly used for residential pest control (Atwood and Paisley-Jones 2017). The half-life of carbaryl is 10 days at a pH of 7 and it has been found at concentrations up to 1.5 mg/L in aquatic ecosystems (Norris et al. 1983). Carbaryl has documented lethal (LC50 values ranging from 1.2 to 22 mg/L) and sublethal effects (e.g., altered activity levels, reduced predator avoidance behavior) on wood frogs and other amphibians (Boone and Bridges 1999, Bridges 1999, Bridges and Semlitsch 2000, Rohr et al. 2003)

Animal collection and husbandry

We collected wood frog egg masses from 8 populations (range: 7 – 10 masses/population) in northwestern Pennsylvania from 31 March to 7 April 2019. All

populations were separated by >5 km, well beyond the demonstrated ~1 km genetic neighborhood of wood frogs (Berven and Grudzien 1990, Semlitsch 2000). The ponds used in this study varied in their proximity to agriculture, which has been shown to correlate with pesticide tolerance in larval wood frogs (Cothran et al. 2013, Hua et al. 2013, 2015b). Distance to agriculture was measured as the linear distance from each pond to the nearest agricultural field using Google Earth (2019, v. 7.3.2) Additional details for how proximity to agriculture was measured are presented in Hua et al. (2015). After collection, I transported the egg masses to the Purdue Wildlife Area in West Lafayette, IN. I distributed the egg masses from each population into 180 L outdoor culturing pools filled with aged well water, keeping eggs from each population separate. After hatching, tadpoles were fed rabbit chow (Purina) *ad libitum*. Tadpole health was checked daily until the start of experiments.

Phase 1: Sublethal pesticide exposures

On 9 May 2018, I transferred a subset of wood frog tadpoles from each population to the laboratory to acclimate to indoor conditions for 24 hr (12:12 light cycle, 23°C). I euthanized (MS-222 overdose) and preserved 10 haphazardly selected tadpoles to assess differences in size and development among populations prior to the start of the experiment. On 10 May 2018, I haphazardly chose 200 individuals from each population and assigned them to one of two pesticide treatments: 1 mg/L carbaryl or pesticide-free control (UV-irradiated, carbon-filtered well water). These two groups represented the pesticide-exposed and pesticide-naïve tadpoles, respectively. I used 14-L plastic containers filled with 7 L of treatment solution as the experimental units and assigned 100 individuals from each population to both treatments. Each treatment was replicated four times per population for a total of 64 experimental units (N = 25 tadpoles/container). I chose 1 mg/L as the nominal carbaryl concentration as this concentration

has been shown to induce a plastic increase in pesticide tolerance without causing mortality (Hua et al. 2014). I made a 20 g/L stock solution by dissolving technical grade carbaryl in 100% ethanol. I then added 350 μL of carbaryl stock solution to the appropriate experimental units. Sorption of carbaryl by plastic experimental units is minimal (approx. 0.1%; Bridges 2000). I then added 350 μL of 100% ethanol to each pesticide-free control experimental unit (0.005%) as sham exposures to account for solvents. The carbaryl solution was not renewed and I conducted no water changes over the 5-d exposure period. On 15 May 2018, tadpoles were transferred into clean 14-L bins filled with 7 L of UV-irradiated, carbon-filtered well water. Tadpoles were maintained in these containers for 2-d prior to the beginning of the parasite exposures. Tadpoles were fed rabbit chow *ad libitum* during Phase 1 and no mortalities were observed.

Phase 2: Parasite exposures

I investigated the effects of pesticide exposure on the susceptibility of tadpoles to two common amphibian parasites via two separate parasite exposure experiments. Prior to the start of the parasite exposures on 17 May, I homogenized the tadpoles from each treatment by population and then haphazardly selected individuals for each experiment. For both experiments, experimental units were 130-mL cups filled with 100 mL UV-irradiated well water. Each experimental unit was assigned one tadpole. The units for the experiments were housed on two separate shelving units in the same room. On each shelving unit, cups were randomly assigned a location on one of the three vertical shelves, with an equal number of cups from each treatment per shelf. I fed all tadpoles rabbit chow *ad libitum* throughout the experiments.

Trematode experiment

To obtain echinostome cercariae, I collected ~100 adults ramshorn snails (*Helisoma trivolvis*), the first intermediate host of echinostome cercariae, from a large permanent pond at

the PWA on 4 May 2018. To screen the snails for infection, I isolated them individually in 50-mL tubes filled with 35 mL of UV-irradiated well water and placed them 10 cm under a light source (100 W light bulbs) for 1-h to induce cercarial shedding (Szuroczki and Richardson 2009). I identified echinostome-infected snails (n =25) by placing cercariae on slides under a compound scope following Schell (1985). Although I did not identify the cercariae from each snail to species using molecular methods, previous work has identified two echinostome species in the PWA pond: *Echinostoma trivolvis* and *Echinoparyphium* lineage 3, with *E. trivolvis* as the more abundant species (L. S. Billet, personal observation; Hua et al. 2016). To reduce shedding prior to the experiment, I individually isolated infected snails in 2 L cups filled with 1.5 L UV-irradiated well water and stored them at 7°C. Two days prior to the experiment, the snails were acclimated to 23°C. Snails were fed a mixture of rabbit chow and spirulina powder *ad libitum*.

I haphazardly chose 25 tadpoles from each phase 1 treatment for each population. On 17 May 2018, I individually exposed 20 of the tadpoles from each treatment per population to echinostome cercariae for a total of 16 treatments and 320 experimental units. The remaining 5 tadpoles were assigned as no-trematode controls to assess background mortality (80 additional experimental units). I used an aliquot method to administer the cercariae (Tucker et al. 2001, Buss and Hua 2018). To do this, I first shed the 25 echinostome-infected snails individually in 50 mL tubes filled with 35 mL of UV irradiated well water under a light source for 1 h. I then checked that each snail was shedding echinostome cercariae under a dissecting microscope and homogenized the water from each tube in a 1-L beaker. To quantify cercarial density, I gently mixed the water and took five 1 mL subsamples. The subsamples had an average of 28.8 cercariae/mL ± 3.27 SD. Based on this, I dispensed 2 mL of cercariae slurry into each experimental unit for an approximate density of 57.6 cercariae/experimental unit. I used the

aliquot method of counting to ensure that cercariae were added to experimental units within 2 h of shedding to control for cercarial age between experimental units.

I checked for tadpole mortality daily, and no water changes were conducted throughout the experimental exposure. I ended the experiment after 3 days (20 May), as this is enough time for echinostome cercariae to successfully encyst in the kidneys as metacercariae without allowing parasite clearance to begin (Hoverman et al. 2013). I euthanized (MS-222 overdose) and preserved tadpoles in 10% buffered formalin. I weighed, staged, and measured snout-vent length and total length of individuals prior to dissection. To quantify trematode load, I first dissected the kidneys of each tadpole, placed them between two microscope slides, and then counted the total number of metacercariae under a compound microscope (Schotthoefer et al. 2003). I then examined the rest of the body for metacercariae; however, all cysts were found in the kidneys.

Ranavirus experiment

The ranavirus exposure experiment began on 17 May 2018. I used a ranavirus strain isolated from an infected green frog (*Rana clamitans*) at the PWA. The virus was cultured on fathead minnow cells and Eagle's minimum essential media (MEM) containing 5% fetal bovine serum to a titer of 2.07×10^6 plaque-forming units [PFU]/mL.

I conducted a factorial experiment that crossed 20 tadpoles from each phase 1 treatment, by population, with either the presence or absence of ranavirus exposure (10^3 or 0 PFU/mL) for a total of 32 treatments and 640 experimental units. To achieve a concentration of 10^3 PFU/mL, I added 48 μL of the virus to each experimental unit assigned to the virus treatment. I then added 48 μL of MEM to each experimental unit assigned to the control treatment.

I conducted mortality checks to determine if virus-induced mortality rates differed by treatment and population. Checks were conducted daily prior to 23 May 2018 and every 6 hours beginning at 6:00 AM on 23 May. At each check, dead individuals were preserved individually in 70% ethanol and their time-to-death (TTD) was recorded. Water changes were conducted every 3 days. The experiment was terminated on 30 May 2018 at 6:00 AM after 12 days, which is sufficient time to observe disease outcomes from ranavirus exposure in wood frogs (Hoverman et al. 2011). I euthanized all surviving individuals and individually preserved them in 70% ethanol to quantify infection status and viral load.

I weighed, staged, and measured snout-vent length and total length of individuals prior to dissection. To quantify infection status and viral load, I dissected the liver and kidneys of each tadpole, combined the tissues in a 1.5 mL microcentrifuge tube, and stored the sample at -80 °C for later DNA extraction and qPCR analysis. To prevent cross-contamination, all tools were soaked in 10% bleach and gloves were changed between dissections.

I conducted DNA extractions using DNeasy Blood and Tissue Kits (Qiagen). I used quantitative polymerase chain reaction (qPCR) to determine infection status and viral load of individuals following the methods of Wuerthner et al. (2017). Each reaction contained 6.25 µl of SsoAdvanced™ Universal Probes Supermix (Bio-Rad Laboratories), 0.1125 µl of forward and reverse primers at 10 pmol/µl each (rtMCP-F [5′- ACA CCA CCG CCC AAA AGT AC- 3′] and rtMCP-R [5′- CCG TTC ATG ATG CGG ATA ATG- 3′]), 0.0313 µl of fluorescent probe rtMCP-probe (50- CCT CAT CGT TCT GGC CAT CAA CCA-30), 3.49 µl of reverse osmosis water, and 2.5 µl of template to a final volume of 12.5 µl. I used a CFX Connect™ (Bio-Rad Laboratories) to conduct qPCR. I included a standard curve and a negative control containing reverse osmosis water as template. I used a synthetic double-stranded DNA standard by

synthesizing a 250- bp fragment of the major capsid protein (MCP) gene (gBlocks Gene Fragments; Integrated DNA Technologies), which is conserved among *Ranavirus* species. I prepared a log-based dilution series ($4.014 \times 10^7 – 4.014 \times 10^4$ viral copies/µl) for the standard curve. Each standard curve sample and unknown sample were run in duplicate. For each unknown sample, I calculated viral load (viral copies ng^{-1} DNA) by dividing the number of copies of ranavirus DNA (viral copies μl^{-1}) by the total DNA present in the sample (ng DNA μl^{-1}). All duplicate unknown samples that peaked before cycle 40 were considered positive. I did not detect discrepancies between replicates (i.e. both replicates were either positive or both were negative).

Pesticide analysis

I collected one 25-mL water sample from each of the experimental units the day they were dosed with carbaryl to verify pesticide concentrations from the phase 1 exposures and stored them at -20 °C prior to analysis. Briefly, each pesticide sample was spiked with a known concentration of Internal standard (carbaryl-d7), extracted by pouring through a 3 cc Oasis SPE cartridges (Waters INC), and then eluting with 3 mL of acetonitrile prior to delivering to Purdue Bindley Bioscience Center (West Lafayette, IN) for confirmation. One sample was lost prior to analysis (RMD replicate 1). The mean carbaryl concentration of the 23 samples from the phase 1 treatments was 0.94 mg/L ± 0.19 SD (range: 0.56 – 1.34 mg/L).

Statistical analysis

I performed all statistical analyses using R version 3.5.1 (R Core Team 2018). I applied a logarithmic transformation to measures of distance to agriculture. I examined Pearson correlation coefficients between individual-level variables prior to modeling to assess collinearity. Because snout-vent length (SVL), total length, stage, and mass tended to be correlated (Pearson

correlation coefficients > 0.70), I included only SVL in models where appropriate. Because transforming SVL did not improve normality, it was left untransformed for analyses. For each analysis, I assessed if there were population-level differences in disease outcomes. If I found that there were significant population-level differences in disease outcomes, I conducted a second set of analyses to assess if these disease outcomes were related to distance to agriculture, which correlates with pesticide tolerance in this system (Cothran et al. 2013, Hua et al. 2013, 2015b). For the models with distance to agriculture as the predictor variable, I included population as a random factor. For each set of analyses, I compared several models with different combinations of fixed effects and selected the best-fit model by comparing the Akaike Information Criterion (AIC) and selecting the model with the lowest AIC (Nakagawa and Cuthill 2007). I used the Anova() function in the 'car' package to estimate p-values (Fox and Weisberg 2011). Tukey posthoc tests were used to determine significant differences among the populations with the cld() function in the 'multcomp' package (Hothorn et al. 2008).

For the trematode experiment, I analyzed trematode load (number of metacercarial cysts per host) with respect to population using a GLM with a negative binomial distribution using the glm.nb() function in the 'MASS' package (Venables and Ripley 2002), and with respect to distance to agriculture using a GLMM with a negative binomial distribution to account for overdispersion using the 'glmmADMB' package (Fournier et al. 2012). I did not analyze tadpole survival data because only 3 of 320 individuals (0.92%) died.

For the ranavirus experiment, I conducted three separate analyses for infection status, time-to-death, and infection load. I first analyzed differences in infection prevalence with respect to population using a GLM with a binomial distribution (1 = infected, 0 = uninfected) using the glm() function, and with respect to distance to agriculture using a GLMM with a binomial

distribution with the glmer() function in the 'lme4' package (Bates et al. 2015). Second, I analyzed time-to-death with respect to population using Cox proportional hazards models with the coxph() function in the 'Survival' package (Therneau 2015). Because the populations did not significantly vary, we did not analyze differences in survival with respect to distance to agriculture. Third, I analyzed ranavirus load (ln-transformed) of infected tadpoles with respect to population using a GLM with the glm() function, and with respect to distance to agriculture using a GLMM with the lmer() function in the 'lme4' package. For ranavirus load, I conducted two separate analyses to account for differences in viral load between individuals that survived and those that died, as individuals that died had significantly higher viral loads. Uninfected individuals were excluded from analyses. Because the two ranavirus-exposed individuals that died in one population (BOW, control phase 1 treatment) did not have a detectable ranavirus infection, this population was excluded from the analysis of dead individuals.

Finally, I tested for a correlation between population susceptibility to trematode infection and ranavirus infection outcomes using the cor.test() function. The first analysis assessed the correlation between average trematode load and ranavirus infection prevalence among the eight populations. The second analysis assessed the correlation between average cyst count and average viral load of survivors. I did not assess the correlation between average cyst count and average viral load of dead individuals, as there was little variation among populations (range: 16.4 – 17.3 viral copies/ng of DNA).

3.4 Results

Trematode experiment

I had 99.1% survival in the trematode-exposed individuals and 100% survival in the no-trematode control individuals. The average trematode load across treatments ranged from 22.50

to 35.60 (~39.1 – 61.8% encysted). I found a significant effect of population on trematode load ($F_{7,310} = 2.2$, $P = 0.033$; Fig. 3.1) as well as a significant negative relationship between a population's distance to agriculture and trematode load ($F_{1,314} = 4.6$, $P = 0.034$; Fig. 3.2). I found a significant positive effect of snout-vent length on trematode load in both analyses ($F_{1,310} = 29.6$, $F_{1,314} = 40.2$, $P < 0.001$). I did not, however, find a significant effect of pesticide exposure on trematode load in either analysis ($F_{1,310} = 0.11$; $F_{1,314} = 0.13$; both $P = 0.7$).

Ranavirus experiment

I had 100% survival in the no-virus control individuals. Therefore, they were excluded from analyses. The infection prevalence, proportion of infected individuals surviving, and average ln viral load of infected individuals across populations and treatments ranged from 30 – 90%, 0 – 64%, and 11.3 – 15.9 ln viral copies/ng of DNA, respectively.

The first set of analyses assessed differences in infection prevalence following ranavirus exposure. I found a significant effect of population ($F_{7,303} = 3.4$, $P = 0.002$) and a significant interactive effect between population and pre-treatment ($F_{7,303} = 3.1$, $P = 0.004$) on infection prevalence (Fig. 3.3). However, there was no effect of a population's distance to agriculture on infection status ($\chi^2 = 0.75$, $P > 0.05$). I also found no main effect of pesticide exposure on infection prevalence in both analyses ($F_{1,303} = 0.02$, $P > 0.05$). There was a significant negative effect of snout-vent length on infection status in both analyses ($F_{1,303} = 20.7$, $P > 0.05$).

The second set of analyses assessed differences in survivorship following ranavirus exposure. There was no significant effect of population or phase 1 treatment on the likelihood of mortality ($\chi^2 = 7.4$, $P > 0.05$). As expected, there was a significant effect of infection status on the likelihood of mortality ($\chi^2 = 30.8$, $P < 0.001$).

The final set of analyses assessed differences in viral load following ranavirus exposure. For infected individuals that survived ranavirus exposure, I found a significant effect of population on viral load ($F_{7,118} = 3.3$, $P = 0.003$; Fig. 3.4); however, there was no effect of a population's distance to agriculture on ranavirus load for survivors ($\chi^2 = 0.2$ $P > 0.05$). There was also no effect of pesticide treatment on viral load of survivors ($F_{1,118} = 1.7$, $P > 0.05$). For individuals that died from ranavirus exposure, I found no significant effect of population ($F_{6,70} = 2.0$, $P > 0.05$) or pesticide treatment on viral load ($F_{1,70} = 0.9$, $P > 0.05$).

Comparisons of pathogen susceptibility

I found no significant correlation between average trematode load and either ranavirus infection prevalence or viral load of survivors (all $P > 0.05$).

3.5 Discussion

Using eight wood frog populations, I explored how exposure to the insecticide carbaryl differentially influenced infection outcomes. I did this by exposing tadpoles to the pesticide carbaryl (1 mg/L) or a no-pesticide control, followed by exposure to one of two common amphibian parasites. I found that there was significant population-level variation in trematode susceptibility, ranavirus infection prevalence, and viral load of survivors. Moreover, I found that the variation in trematode susceptibility was related to a population's proximity to agriculture, with tadpoles from populations located close to agriculture being more susceptible to trematode infections compared to individuals from populations farther from agriculture. However, I found no significant effects of carbaryl exposure on disease outcomes.

Contrary to predictions, I found little effect of pesticide exposure on trematode susceptibility relative to control individuals. In other words, infection outcomes were relatively consistent within a population, regardless of the pesticide exposure history. This is surprising

given that wood frogs are sensitive to carbaryl (Boone and Bridges 1999, Bridges 1999, Bridges and Semlitsch 2000, Rohr et al. 2003) and that other studies have demonstrated that prior exposure to 1 mg/L carbaryl affects susceptibility to parasitic infection in other amphibian species (Pochini and Hoverman 2017). The overall echinostome infection success (~51% encystment rate) was high relative to other studies (Holland et al. 2007, Orlofske et al. 2013, Hua et al. 2017), which could indicate that either these wood frog populations are highly susceptible to echinostome cercariae, or that the cercariae used in this study were particularly infective. Based on the difference in mean echinostome infection success in three populations used in both the present study and a previous study by Hua et al. (2017) (~57.8% vs ~33.3%), it is more likely the latter. Alternatively, high infection rates could be due to the size of the experimental units. Because each tadpole in the experiment was housed in a relatively small volume of water (100 mL), the experimental design limited the effectiveness of behavioral parasite avoidance, an important strategy for preventing trematode infections (Koprivnikar et al. 2006a). Thus, the study may be more representative of differences in host immune resistance between groups. The results of this study suggest that carbaryl exposure of up to 1 mg/L does not significantly alter the tadpole immune response to trematodes. However, because behavioral avoidance can play a prominent role in preventing echinostome infections and carbaryl has been shown to alter tadpole behavior (Bridges 1997, Koprivnikar et al. 2006a, Daly and Johnson 2011), future studies should address how a sublethal carbaryl dose can influence behavioral responses to parasites and how that influences infection success.

Although I found no effect of pesticide exposure on trematode susceptibility, I did find significant population-level variation in trematode susceptibility, with a 33.8% higher infection load in the most susceptible population compared to the least susceptible. This is consistent with

previous studies, which have found significant population-level variation in echinostome infection across wood frog populations (Hua et al. 2017). I also found that this variation in trematode susceptibility was related to a population's distance to agriculture, with individuals from populations close to agriculture being more susceptible to infection with echinostome trematodes than individuals from populations far from agriculture. Interestingly, these findings differ from those of Hua et al. (2017), which found that populations closer to agriculture were less susceptible to infection with echinostome trematodes than populations far from agriculture. However, the previous study was conducted with 15 wood frog populations with relatively well-established measures of constitutive tolerance and inducible tolerance (see Hua et al. 2015a), whereas the present study was conducted with eight populations with limited measures of pesticide tolerance. Considered together, these results suggest that linear distance from a pond to the nearest agricultural field, while informative in some respects, provides an incomplete picture of interpopulation pesticide tolerance strategies and that other factors that may influence pesticide tolerance (i.e., biotic and abiotic stressors) should be considered.

I found that, consistent with past work, larval wood frogs are highly susceptible to ranavirus, with 65.6% (n = 210) of individuals that were exposed becoming infected. Although I found no significant main effect of pesticide exposure on ranavirus infection outcomes, there were significant differences in infection prevalence among the eight populations (range: 42.5% - 80.0%). This demonstrates that intraspecific variation in infection susceptibility can be nearly as dramatic as interspecific variation (Hoverman et al. 2011). Moreover, I found a significant interaction between population and carbaryl exposure on ranavirus infection prevalence. The interaction was driven mainly by two populations, one which had significantly higher infection prevalence in the pesticide-exposed group (LOG) and one which had significantly lower

infection prevalence in the pesticide-exposed group (MAP). This highlights the complex influence that pesticides can have on host-parasite interactions and how variable this influence can be between populations. Additionally, I found that there was significant population-level variation in viral load of survivors (range: 9.3 – 14.0 ln viral copies/ng of DNA). I found little population-level variation in viral load of dead individuals (range: 16.4 – 17.3 ln viral copies/ng of DNA), consistent with previous studies that demonstrate that amphibian mortality is highly likely beyond a threshold viral load (Wuerthner et al. 2017, Hua et al. 2017). Together, these results suggest that although wood frogs are highly vulnerable to ranavirus infection (Hoverman et al. 2011), populations can vary widely in susceptibility and infection outcomes. It remains unclear, however, what factors drive these population-level variations in virus susceptibility. Future studies should explore whether differences in innate or adaptive immunity influence these interpopulation differences in virus susceptibility and if there are differences in biotic or abiotic stressors between sites that drive these differences.

I found no correlation between average trematode load and either ranavirus infection prevalence or average viral load of survivors, underscoring that populations of the same species can be differentially susceptible to different pathogens and that the underlying physiological mechanisms resulting in higher resistance to one pathogen do not necessarily incur higher resistance to another. In the context of this study, the parasites used are functionally different (macroparasite vs microparasite), making it is unlikely that the selection for resistance to one would incur higher resistance to the other. Moreover, the immune responses to these two parasites differ greatly; whereas exposure to the more virulent pathogen, ranavirus, can induce a strong adaptive immune response in anurans (Gray et al. 2009), exposure to the more benign pathogen, echinostomes, has been shown to elicit a relatively weak physiological response

(Koprivnikar et al. 2019). Although correlated selection is unlikely, coinfection with these two pathogens can alter infection outcomes by reducing virus replication rates, suggesting that cross-reactive immunity can occur between these two pathogens and that the immune response to one can influence the immune response to the other (Wuerthner et al. 2017).

While it is well-documented that responses to pesticides and parasites vary among amphibian species (Bridges and Semlitsch 2000, Hoverman et al. 2011, Gahl et al. 2012, Hammond et al. 2012, Gervasi et al. 2013, 2017, Blaustein et al. 2018), there is considerably less literature experimentally exploring this variation at the population-level on a relatively small geographic scale. The goal of this study was to explore how exposure to the insecticide carbaryl differentially influences parasite infection outcomes among wood frog populations. While I found little evidence that carbaryl exposure influenced parasite susceptibility, I did find evidence for substantial interpopulation differences in susceptibility to two common amphibian parasites, ranavirus and echinostomes. Moreover, I found that differences in trematode susceptibility were related to a population's distance to agriculture, with individuals from populations close to agriculture being more susceptible to infection with echinostome trematodes than individuals from populations far from agriculture. This study adds to this growing literature and provides evidence that populations can vary significantly in susceptibility to pathogens, and that high susceptibility to one common pathogen does not necessarily predict susceptibility to another (Pearman and Garner 2005, Koprivnikar et al. 2006b, Bradley et al. 2015, Hua et al. 2017). Further research is needed to parse out how environmental differences among populations, including land-use patterns, as well as evolutionary history impact susceptibility to parasites, and if these patterns of susceptibility are consistent across space and time. To this end, long-term

studies that track population-level variation in parasite susceptibility through time will greatly increase our understanding of these trends.

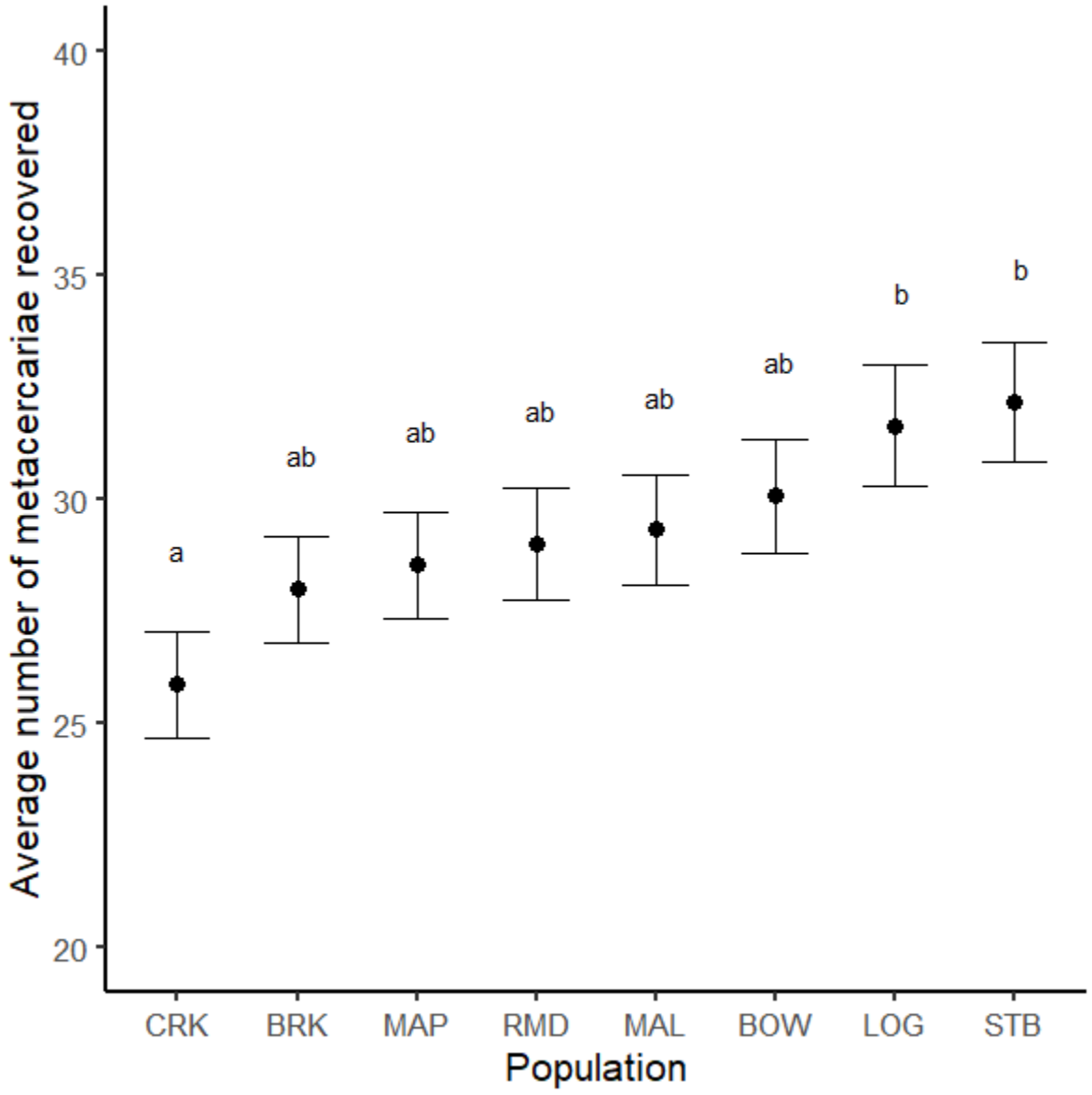

Figure 3.1. Number of metacercariae encysted in tadpoles (estimated marginal means ± SE) by population (represented by three letter abbreviation), without regard to previous pesticide exposure. Populations sharing lower case letters are not significantly different from one another ($P > 0.05$).

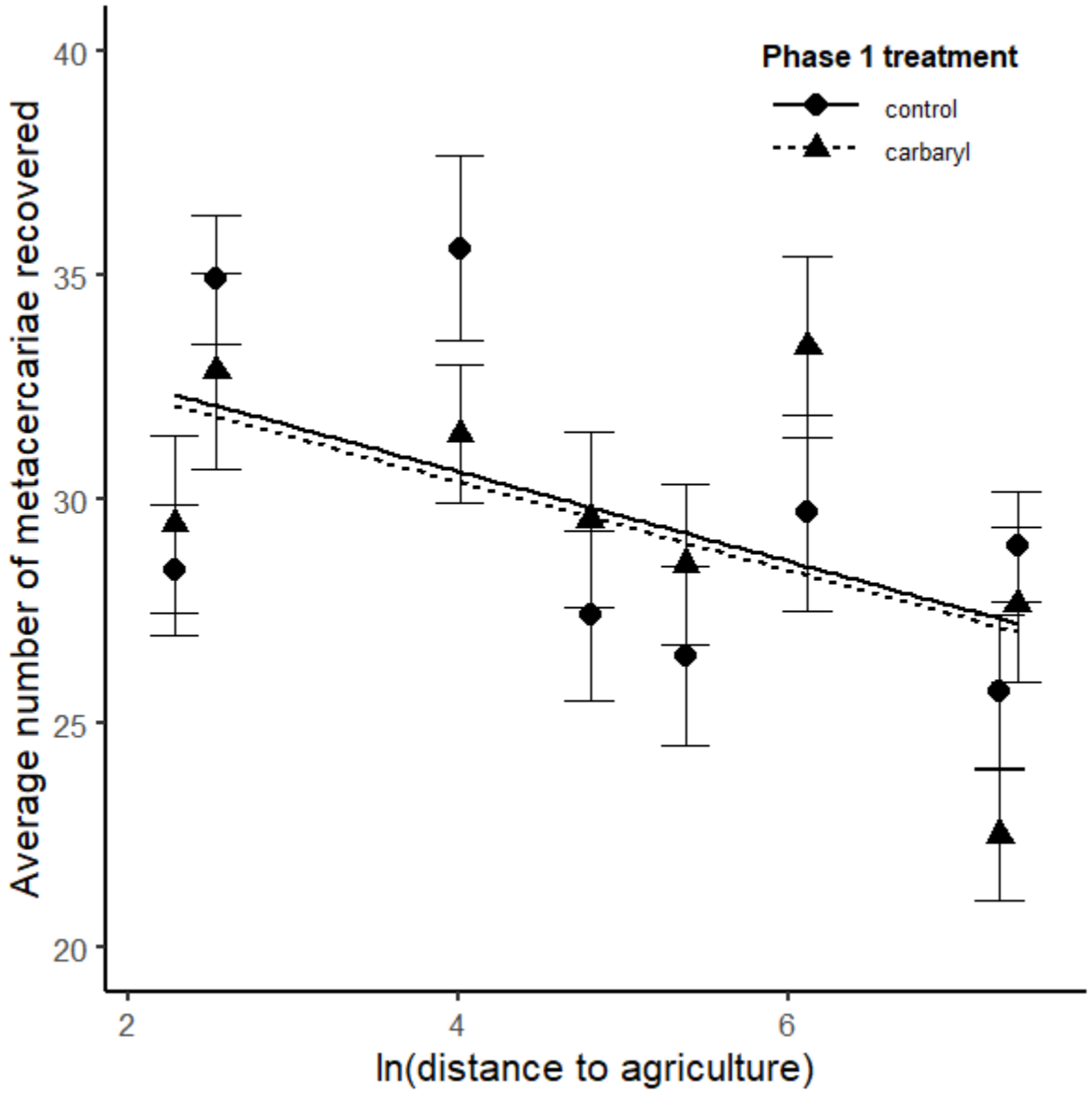

Figure 3.2. Number of metacercariae encysted in tadpoles (mean ± SE) by population distance to agriculture (ln-transformed) with regard to pesticide treatment (control = circles, solid line; 1 mg/L carbaryl = triangles, dotted line).

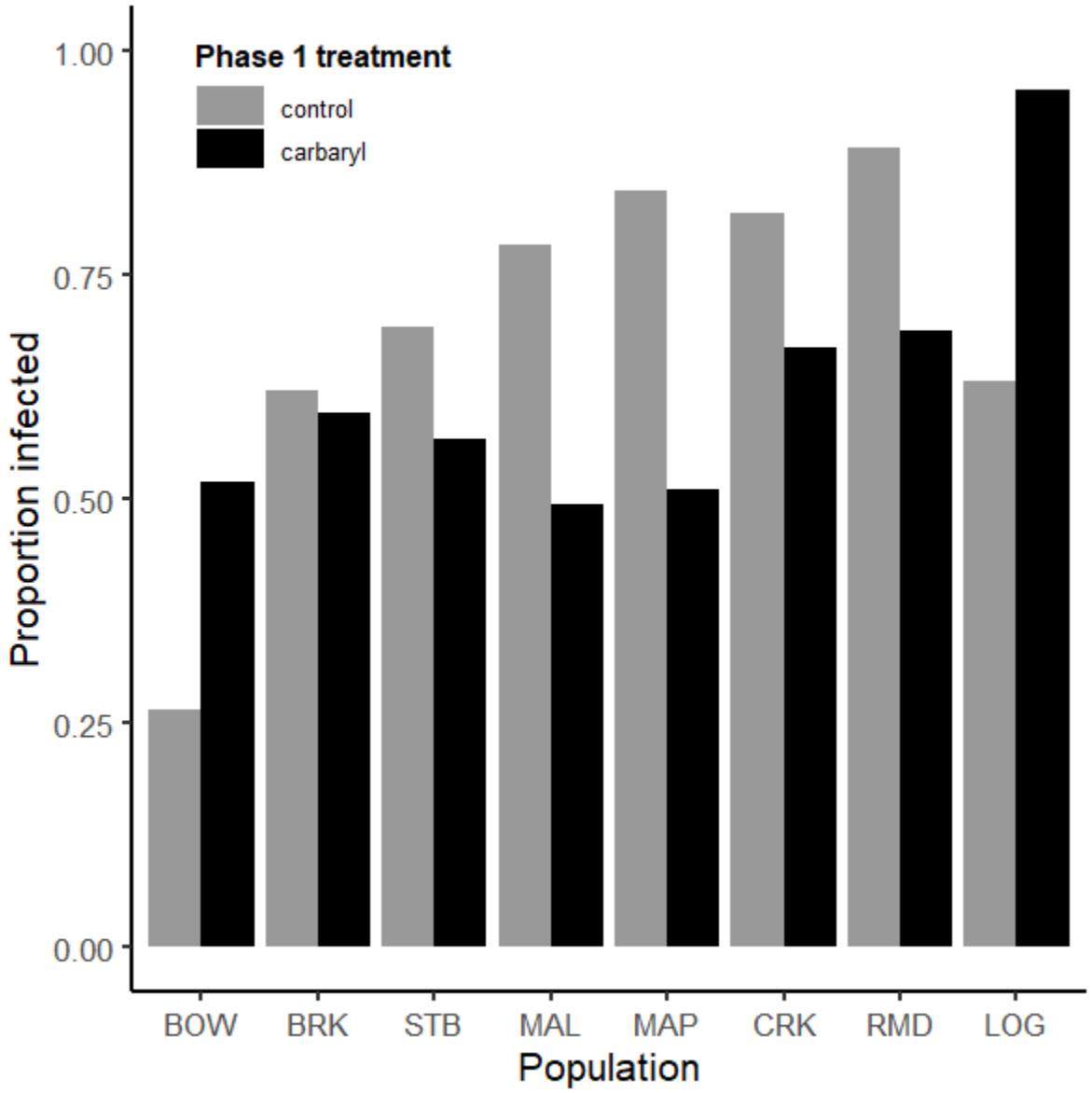

Figure 3.3. Ranavirus infection prevalence (estimated marginal means) by population (represented by three letter abbreviation), with respect to phase-1 treatment (control = gray bars; 1 mg/L carbaryl = black bars).

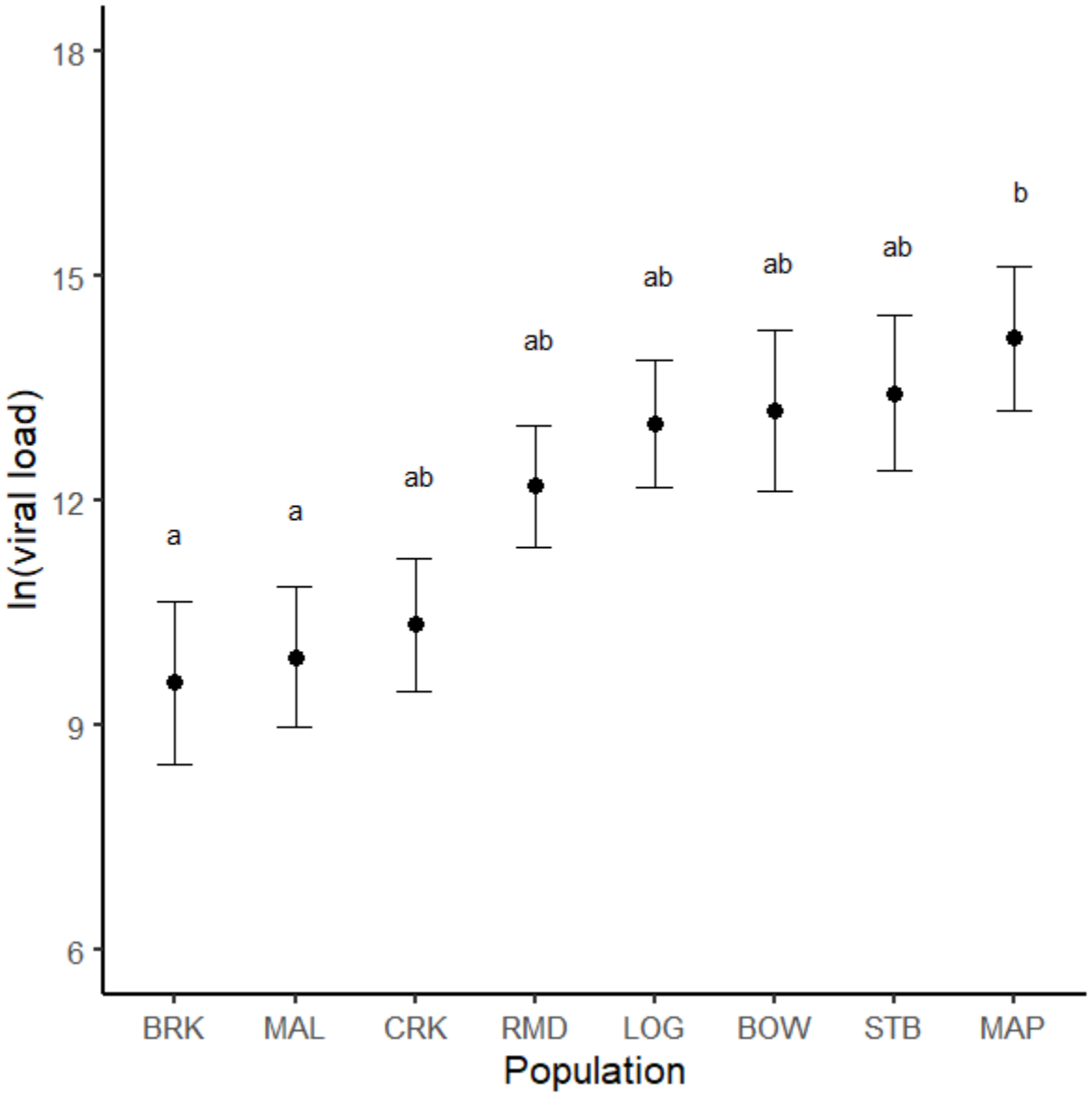

Figure 3.4. Viral load (ln-transformed, estimated marginal means±SE) by population (represented by three letter abbreviation), without regard to previous pesticide exposure. Populations sharing lower case letters are not significantly different from one another (P > 0.05

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