

12-2010

# Impacts of predation risk and development on susceptibility of North American anurans to ranaviruses

Nathan Alden Haislip
*University of Tennessee, Knoxville*, nhaislip@utk.edu

To the Graduate Council:

I am submitting herewith a thesis written by Nathan Alden Haislip entitled "Impacts of predation risk and development on susceptibility of North American anurans to ranaviruses." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Wildlife and Fisheries Science.

Matthew J. Gray, Major Professor

We have read this thesis and recommend its acceptance:

Jason T. Hoverman, Debra L. Miller, Arnold Saxton

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

To the Graduate Council:

I am submitting herewith a thesis written by Nathan Alden Haislip entitled "Impacts of predation risk and development on susceptibility of North American anurans to ranaviruses." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Wildlife and Fisheries Science.

_____
Matthew J. Gray, Major Professor

We have read this thesis
And recommend its acceptance:

_____
Debra L. Miller

_____
Arnold M. Saxton

_____
Jason T. Hoverman

Accepted for the Council:

_____
Carolyn R. Hodges
Vice Provost and Dean of the
Graduate School

# Impacts of predation risk and development on susceptibility of North American anurans to ranaviruses

**A Thesis**
**Presented for the**
**Master of Science**
**Degree**
**The University of Tennessee, Knoxville**

**Nathan Alden Haislip**
**December 2010**

## ACKNOWLEDGEMENTS

I would like to start by thanking Dr. Matt Gray. I have learned an immense amount from Matt ever since he first began mentoring me as an undergraduate. Through the completion of my M.S., he played an integral role in my professional development and career. I also greatly appreciate the time, hard work, and dedication of other members of my graduate committee: Dr. Jason Hoverman, Dr. Debra Miller, and Dr. Arnold Saxton. Their input and support helped to shape my ideas into sound experiments. I would like to especially thank Dr. Hoverman. Jason has been a second advisor to me, and without his insight, guidance, and support my research would not have been completed.

I want to thank the Tennessee Wildlife Resources Agency, the University of Tennessee Institute of Agriculture, and the University of Georgia Veterinary Diagnostic and Investigational Laboratory (VDIL) in Tifton for providing funds and in-kind support for my research. I am grateful for the staff at the East Tennessee Research and Education Center for providing lab space and logistical support for conducting my research; thank you Drs. Hodges, Simpson, and Campbell, and thank you Roger. I am indebted to the Tifton VDIL for providing the virus isolate and conducting the real-time PCR, especially Dr. Baldwin, Dr. Miller, Lisa Whittington and Dallas Ingram.

I would also like to thank Nathaniel Hilzinger, Heather Edwards, Lacy Rucker, Billy Thompson, and Erica Haislip for providing assistance with the experiments. These individuals worked countless hours; their assistance was vital to me completing my research. Thank you especially, Nate, for all your help with DNA extractions and running real-time PCR! Lastly, I would like to thank all my family and friends for their enduring support and guidance. I greatly appreciate you all!

## ABSTRACT

For over three decades, amphibian populations have been declining across the globe. Emerging infectious diseases are responsible for some of these declines. Ranaviruses have caused die-offs in wild amphibian populations on 4 continents, in 5 Canadian provinces, and in over 25 U.S. states. In order to understand host-pathogen dynamics, it is critical to establish baseline information on species susceptibility and the effects of natural stressors. The goal of my thesis research was to quantify the effects of anuran development and exposure to invertebrate predators on species-specific susceptibility to ranavirus. My experiments were designed in factorial arrangements, and consisted of exposure to ranavirus during different developmental stages or with and without predator cues in a controlled environment. I found that exposure to invertebrate predator cues did not increase susceptibility to ranavirus for 4 anuran species tested. Susceptibility differed among embryo, hatchling, larval and metamorph stages, but trends differed among species and did not follow predictions based on *Xenopus laevis* immune function. Low susceptibility during the embryo stage was the only consistent development result among species, perhaps owing to protective qualities of the vitelline membrane or mucoidal capsules surrounding the embryo. Across 7 anuran species tested, mean mortality rates ranged from 5 – 100%, with *Lithobates sylvaticus* and *Scaphiopus holbrookii* most susceptible. I found that infection rates and viral load were correlated with mortality rates, thus these variables are good indicators of susceptibility to ranavirus. My results indicate that ranaviruses can cause catastrophic natural mortality in some anuran species, and likely play a significant role in local population dynamics. For highly susceptible species, ranaviruses could cause local extirpations that lead to species declines. More information is needed on the role of natural (e.g., co-infection, competition) and anthropogenic stressors in driving ranavirus epizootic events. I encourage natural resource agencies to initiate ranavirus surveillance programs, especially for rare species and fragmented populations. Future studies should take an immunogenetic approach to identifying mechanisms driving susceptibility. Identifying mechanisms associated with ranavirus emergence is fundamental to developing science-based conservation strategies.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

# CHAPTER I

## INTRODUCTION

Since the 1970s, mass mortality events of amphibians have been reported and recent evidence suggests that 43% of amphibian species are in decline, with 32% of species listed in threat of extinction (Carey 2000, Stuart et al. 2004). In the 1990s, two pathogens, *Batrachochytrium dendrobatidis* (*Bd*) and Iridoviruses in the genus *Ranavirus*, emerged as considerable threats to amphibian populations (Cunningham et al. 1993, Longcore et al. 1999). While both pathogens have been linked to mortality events in North America, Central America, South America, Europe, and Asia (Carey et al. 2003a), the majority of reported mortality events in the United States have been associated with ranaviruses (Green et al. 2002, Muths et al. 2006). In 96% of these reports, larvae and recently metamorphosed individuals experienced the greatest mortality, although substantial loss of adult amphibians in the wild has been reported in the United Kingdom and Denmark (Cunningham et al. 1993, Drury et al. 1995, Ariel et al. 2009, Teacher et al. 2010).

Relatively few studies have been conducted to determine the effects of ranavirus in combination with natural or anthropogenic stressors. Amphibians are exposed to a variety of ecological stressors, which can decrease immune function. For example, Denver (1997) demonstrated that *Scaphiopus couchii* tadpoles accelerated development in response to habitat desiccation, which was mediated through endogenous production of a corticotropin-releasing hormone that stimulated glucocorticoid production. Belden and Kiesecker (2005) found that tadpoles exposed to exogenous corticosterone (i.e., a glucocorticoid) were infected by trematodes at 3X the rate of control tadpoles. Thus, amphibians exposed to stressors may be more susceptible to pathogens.

In Chapter II, I focus on determining the effects of predatory stress on host susceptibility to ranavirus. Amphibians respond to predators by altering their behavior, morphology, and life history characteristics, which often enhances survival (Lima and Dill 1990). However, predators also induce stress in amphibians, which may compromise immune function and increase their susceptibility to pathogens. The objective of this study was to determine if the chronic stress associated with the threat of predation increased the susceptibility of anuran larvae to ranavirus. To accomplish this goal, I exposed tadpoles of 4 anuran species to ranavirus and predator cues from 2 species of aquatic insects that differed in the level of risk posed to amphibians. Chemical cues associated with predation were generated by feeding non-experimental tadpoles to the aquatic insects. I hypothesized that: 1) the combination of predator cues and ranavirus would reduce activity and growth to a greater degree than either factor alone, 2) as predator risk increased tadpole susceptibility to ranavirus (as indexed by viral load and mortality rates) would increase, and 3) anuran species that exhibited stronger stress responses to predation would experience greater susceptibility to ranavirus infection when exposed to both factors.

In Chapter III, I focus on quantifying the effects of larval development on the susceptibility to ranavirus. Extensive literature exists on the development of the immune system in anuran larvae (Fox 1963, Manning and Horton 1969, Du Pasquier and Weiss 1973, Du Pasquier et al. 1989, Hansen and Zapata 1998, Rollins-Smith 1998). These studies indicate that immune system function increases through the embryo and hatchling stages (Gosner [1960] stages 0-25), peaks during the larval stages (Gosner stages 26-41), and decreases during metamorphosis (Gosner stages 42-46). Field evidence at die-off sites suggests that later developmental stages are most susceptible (Green et al. 2002, Carey et al. 2003b); however, these results may be confounded by differences in detecting dead individuals. For example, it is

easier to see a die-off of larvae or metamorphs due to their larger size compared to hatchlings or embryos. Most experimental challenges with ranavirus have focused on larval or metamorph stages (e.g, Gantress et al. 2003, Pearman and Garner 2005, Brunner et al. 2007, Hoverman et al. 2010), although there are a couple studies that exposed embryos to ranavirus (Granoff et al. 1965, Tweedell and Granoff 1968). However, none of these studies compared all 4 developmental stages simultaneously, thus it is unknown if certain stages are more susceptible to ranavirus infection and morbidity. Identifying developmental stages that are most susceptible to ranavirus can help guide pathogen surveillance and population monitoring initiatives. The objective of this study was to determine how susceptibility to ranavirus differed among developmental stages and whether any trends were consistent among species. To accomplish this goal, I exposed tadpoles of 7 anuran species to ranavirus during the 4 aforementioned developmental stages. Based on previous studies, I hypothesized that susceptibility (as indexed by infection rates, mortality rates, and viral load) would be higher at stages 11 and 41 (i.e., embryos and metamorphs) due to early development and suppression of the amphibian immune system during metamorphosis, respectively, compared to stages 21 and 30 (i.e., hatchlings and larvae).

A secondary goal was to determine if species-specific differences existed in susceptibility to ranavirus. There is little information to date on the relative susceptibility of amphibian species to ranaviruses under controlled laboratory conditions (Hoverman et al. 2010). Amphibians in the anuran family Ranidae and caudate family Ambystomatidae often are reported in die-off events (Jancovich et al. 1997, Bollinger et al. 1999, Green et al. 2002, Docherty et al. 2003, Schock and Bollinger 2005), totaling 96% of reported ranavirus-associated mortality events processed by the United States National Wildlife Health Center from 1996 – 2001 (Green et al. 2002). This report

suggests that other amphibian families may have low susceptibility to ranaviral infection, but differences in the likelihood of detecting a die-off among species may be a confounding factor. I hypothesized that differences in susceptibility to ranavirus would vary among species and be related to life history characteristics such as type of breeding habitat or duration of pre-metamorphic development. For example, species that inhabit more permanent wetlands probably have a greater likelihood of exposure to ranavirus virions, because water is an excellent transmission media, environmental persistence of virions in water may exceed 2 weeks, and there are typically a greater number of ectothermic vertebrate reservoirs such as fish and turtles (Gray et al. 2009). If amphibian species in more permanent wetlands have been exposed to ranavirus more frequently over evolutionary time, their innate immune system (e.g., macrophages, neutrophils, natural killer cells) should be more capable of destroying ranavirus virions compared to species that inhabit ephemeral habitats. Additionally, species inhabiting more ephemeral habitats often exhibit shorter larval durations than those of permanent systems. It is possible that anurans in temporary habitats may devote more physiological resources to rapid growth and metamorphosis at the cost of immune function. I discuss species-specific trends in susceptibility in both chapters.

My thesis is written in manuscript style. Thus, each chapter contains Introduction, Methods, Results and Discussion sections at a minimum. I intend to submit Chapter II to *Oikos* or *Oecologia* and Chapter III to *Ecology*.

# CHAPTER II

## COMBINED EFFECTS OF PREDATION RISK AND RANAVIRUS ON FOUR ANURAN SPECIES

## INTRODUCTION

Emerging infectious diseases (EIDs) have sparked concern throughout the scientific community due to the threats posed to global biodiversity (Fowler and Miller 2007, Greger 2007) and, consequently, their impact on the structure and function of ecological communities (Whiles et al. 2006). Numerous EIDs have been linked to anthropogenic factors such as production agriculture, habitat destruction, and global climate change (Greger 2007). Moreover, zoonotic outbreaks such as SARS, influenza, bovine spongiform encephalopathy, and HIV/AIDS have resulted in substantial monetary losses associated with human health expenses, reduced livestock production, and costs of disease prevention and surveillance programs. Given the threats posed by EIDs, studies are needed that address the mechanisms that lead to host susceptibility so that intervention strategies can be implemented to reduce their impacts.

Amphibians are a group of vertebrates that is experiencing population declines and species extinctions (Daszak et al. 1999, Carey 2000, Collins and Storfer 2003). While there are many hypotheses for amphibian die-offs, EIDs are certainly playing a role (Wake and Vredenburg 2008, Collins and Crump 2009). Ranaviruses have caused amphibian die-offs in wild populations on 4 continents (Gray et al. 2009). In North America, known ranavirus-associated die-offs have occurred in over 25 U.S. states and 5 Canadian provinces since 1996 (Gray et al. 2009; Schock et al. 2010; D. E. Green, U.S. Geological Survey, *unpublished data*; M. K. Gahl, University of New Brunswick, *unpublished data*). This pathogen has been associated with nearly 50% of all reported mortality events in North America (Green et al. 2002,

Muths et al. 2006). Mortality rates at die-off sites frequently exceed 90% and often involve larvae or recently metamorphosed individuals (Green et al. 2002). Although ranaviruses have been linked to numerous die-off events, the mechanisms responsible for disease emergence in wild populations remain unclear.

Ecological stressors may be important factors contributing to the emergence of infectious diseases. Stressors are stimuli that activate physiological or behavioral coping mechanisms in organisms that increase survival in the short term (Romero 2004). Despite enhancing short-term survival, stress responses can negatively impact immune functions if they persist in an organism (i.e., chronic stressors; Griffin 1989, Martin 2009). Consequently, persistent ecological stressors that suppress immune function may drive disease outbreaks in wild populations. One common ecological stressor is the threat of predation, which causes prey to adaptively alter their behavior, morphology, and life history traits (Lima and Dill 1990). Research across a diversity of taxa (e.g., invertebates, vertebrates) suggests that predators can negatively impact prey immune function (Boonstra et al. 1998, Rigby and Jokela 2000, Stoks et al. 2006). Thus, a constant threat of predation could increase the susceptibility of an organism to pathogen infection and contribute to the emergence of infectious diseases. If this hypothesis is true, predator-rich environments may be hotspots for pathogen outbreaks.

Amphibians have been used as a model system to describe the ecology and evolution of predator-prey interactions (Wilbur 1972, Morin 1983, Werner 1986). Many species of amphibian larvae have the capability of detecting aquatic predators from chemical cues released during predation and food digestion (Schoeppner and Relyea 2005). These cues can affect activity levels, growth and morphology of larval amphibians (Lawler 1989; Werner and Anholt 1996; Relyea and Werner 1999; Van Buskirk 2001; Relyea 2002a, b). The strength of the

response is often positively correlated with the amount of risk posed (e.g., capture likelihood) by a particular predator species (Relyea 2001a, b).

The specific stress responses of amphibians to predators have not been thoroughly examined. However, in response to other environmental stressors (e.g., habitat desiccation), larval amphibian stress responses are mediated through endogenous production of a corticotropin-releasing hormone that stimulates glucocorticoid production (Denver 1997). Glucocorticoids are known to have immunosuppressive effects in amphibians such as decreasing lymphocyte production and destroying T-cell lymphocytes (Tournefier 1982, Ducoroy et al. 1999), and have been shown to increase susceptibility to trematode infections (Belden and Kiesecker 2005). Thus, it is possible that amphibian responses to aquatic insect predators have immunosuppressive effects that may increase susceptibility to ranavirus infections. To date, no studies have investigated the impacts of the threat of predation on amphibian susceptibility to ranavirus.

The objective of this study was to determine if the chronic stress of predation increases the susceptibility of amphibian larvae to ranavirus. To accomplish this goal, I exposed tadpoles of 4 anuran species to ranavirus and chemical cues generated from 2 species of aquatic insect predators that differed in their level of risk posed to amphibians. I hypothesized that: 1) the combination of predator cues and ranavirus would reduce activity and growth to a greater degree than either factor alone, 2) as predator risk increased, tadpole susceptibility to ranavirus (as indexed by viral load and mortality rate) would increase, and 3) anuran species that exhibited stronger stress responses to predation would experience greater susceptibility to ranavirus infection when exposed to both factors.

## METHODS

### Study Animals and Virus Isolate

For my experiments, I used 4 species of larval anurans (*Lithobates* [*Rana*] *clamitans*, *L. sylvaticus, Pseudacris feriarum*, and *Hyla chrysoscelis*). These species were chosen because they have wide distributions in the eastern United States and they represent the 2 most common families of North American anurans (i.e., Ranidae and Hylidae). Consequently, the results from these species should provide general insights into the effects of predation risk on susceptibility to ranavirus for several closely related species. Additionally, these species vary in their degree of anti-predator responses (Relyea 2001a). For example, *H. chrysoscelis* and *L. sylvaticus* respond more strongly to predators compared to *L. clamitans* (Relyea 2001a), which may equate to differences in predator-induced immunosuppression. No studies have examined the responses of *P. feriarum* to aquatic predators; however, larvae of other *Pseudacris* species are known to respond intermediately to aquatic predators (Skelly 1995, Van Buskirk et al. 1997).

For each species, breeding populations were identified in counties surrounding Knoxville, Tennessee, from January – July 2009. Egg masses were collected for each species (except *H. chrysoscelis*) within 48 hours of deposition, rinsed with sterile water, and transported in 19-L buckets filled with aged tap water to the University of Tennessee Joe Johnson Animal Research and Teaching Unit (JARTU, Table A.4). For *H. chrysoscelis*, amplexed breeding pairs were collected and transported to JARTU for oviposition in covered 11.7-L tub containing 7 L of aged tap water. Breeding pairs remained in containers <24 hrs before oviposition occurred. The day after collection, egg masses were placed outdoors in 300-L wading pools filled with aged tap water to develop. These pools were covered with 60% shade cloth to prevent the colonization of aquatic insects or other amphibians. After hatching, the tadpoles were fed rabbit chow (Purina,

St. Louis, Missouri) and ground TetraMin® (Tetra, Blacksburg, Virginia) *ad libitum* until they were used in the experiments. While vertical transmission of ranaviruses is not known to occur (Gray et al. 2009), we used a random sample of 10 tadpoles from each species to confirm the absence of ranavirus (see Molecular Analyses section); all pre-experiment tadpoles tested negative.

I used larval Aeshnid dragonflies (*Anax* sp.) and adult water bugs (*Belostoma flumineum*) as predators in the experiments. These were collected from farm ponds within 10 km of Knoxville. Once collected, invertebrates were rinsed with sterile water and placed into tubs containing aged tap water in JARTU. These predators are common tadpole predators that represented two different degrees of risk (Relyea 2001a). Aeshnid dragonflies are generally high-risk predators for tadpoles because they have a high prey capture efficiency and short handling time. In contrast, water bugs pose a lower risk due to their poor capture efficiency and long handling time. Consequently, tadpoles tend to exhibit stronger anti-predator responses to chemical cues released during predation by dragonflies compared to water bugs (Relyea 2001b). Before the start of the experiment, the predators were housed individually in 2-L plastic tubs filled with 1 L of aged tap water and fed 1 tadpole per week. Once per week, I conducted water changes to maintain water quality in the tubs. In general, aquatic insect predators were housed and fed for approximately 5 weeks prior to experiments. Similar predator-exposure experiments have maintained and fed aquatic insect predators in captivity for extended durations (Relyea and Auld 2005).

For my experiments, I used a ranavirus that was isolated by the University of Georgia Veterinary Diagnostic and Investigational Laboratory (VDIL) in 2006 from morbid juvenile *L. catesbeianus* housed at a ranaculture facility in southern Georgia (Miller et al. 2007).

Preliminary molecular analyses suggest that the isolate is similar to *Frog virus* 3 (FV3; GenBank accession no. EF101698, Miller et al. 2007). Concurrent research found that the tadpoles species used in my experiments can display signs of disease from this isolate within 1 – 5 days post-exposure (PE) and experience mortality within 5 – 21 days PE, suggesting that the isolate is highly virulent (Hoverman et al. 2010; J. Hoverman, unpublished data). The isolate was cultured at the VDIL using the same protocol described in Hoverman et al. (2010). An aliquot of the stock viral solution was titrated at the VDIL to determine the number of plaque forming units (PFUs). Following titration, the virus was sent overnight to the University of Tennessee and stored at -80°C until used in the experiments. When I received the virus, it was on the third passage since original isolation.

**Experimental Trials**

A separate experiment was conducted for each of the 4 species and all experiments were conducted under identical laboratory conditions (23°C and a 12:12 day:night photoperiod) in JARTU. Each experiment was a factorial combination of 2 virus treatments crossed with 3 predator treatments. The virus treatments included a no-virus control and a virus exposure of $10^3$ PFUs $mL^{-1}$. The predator treatments were a no-predator control and predator cues from either *Anax* or *Belostoma.* Each treatment was replicated 5 times for a total of 30 experimental units. All experiments were conducted at a common shelf height in JARTU. The experimental units were 11.7-L plastic tubs filled with 7 L of aged tap water. Tadpoles that were at Gosner (1960) stage 30 were used to reduce possible confounding effects of development on response variables (see Chapter III). I randomly assigned 10 tadpoles to each experimental unit. An additional random sample of 10 tadpoles was humanely euthanized in benzocaine hydrochloride (1 g $L^{-1}$)

and weighed to the nearest 0.1 mg. The average mass of these tadpoles was used to calculate growth of all tadpoles surviving at the end of the experiment (discussed later).

I used the addition of predator cues, generated during tadpole predation, to simulate predator presence in my experimental units (Fraker 2008). Predator cues consist of kairomones that are released by the predator during prey digestion as well as alarm cues produced by the prey during predation (Schoeppner and Relyea 2005). For each predator species, cues were generated in six 11.7-L tubs filled with 10 L of aged tap water. One day before the start of the experiment, 1 predator was placed into each tub. The predator was housed within a cage constructed of a 250-mL plastic cup with window screen covering the opening. The cage was inverted prior to placing it in the tub so that an air pocket formed, allowing the predator access to surface oxygen. Tadpoles were placed in the cage with the predator to increase the likelihood of consumption and cue generation. The porous screen allowed cues to pass from the cage into the tub.

Predator cues were generated by feeding each predator 400 – 500 mg (1 – 3 individuals) of live tadpoles every day of the same anuran species being tested. The tadpoles were blotted dry and their total biomass recorded prior to addition to the predator cage. Of the 6 tubs per predator species used to generate predator cues, I randomly selected 4 tubs where the predators consumed all offered prey. The 2 extra tubs were maintained to ensure that I had at least 4 predator tubs available for cue generation if some of the predators did not consume prey. I replaced predators that did not eat for 2 consecutive days with new individuals. Also, new individual predators were used between experiments. Predator cue presence was verified by comparing activity levels (discussed below) between cue-exposed and -unexposed tadpoles.

Because the mass of prey consumed by each predator varied, I calculated the predator-cue concentration in each predator tub, which was the ratio of consumed tadpole mass per 10 L of water. I used this value to standardize the cue concentration among predator tubs, with a target cue concentration of 40 mg of tadpole $L^{-1}$. If the calculated cue concentration was greater than the target, I conducted a dilution by mixing together the appropriate amount of aged tap water with the predator-cue water. For example, if the amount of prey consumed by a predator was 500 mg, I added 8 L of predator cue water to 2 L of aged tap water to obtain a final concentration of 40 mg of consumed prey $L^{-1}$. Once the concentrations were adjusted, the 10 L of water from each of the 4 tubs was combined. The mixture was stirred to evenly distribute the cues, and 100 mL distributed to the appropriate predator-cue experimental units. Prior to the cue addition, I removed 100 mL from each experimental unit to compensate for the additional water added to the tub. This resulted in a final concentration of 0.57 mg of consumed tadpole biomass $L^{-1}$ (40 mg $L^{-1}$ × 100 mL = 4 mg added to 7 L = 0.57 mg $L^{-1}$), which was within the cue range (0.071 – 3 mg $liter^{-1}$) known to elicit anti-predator responses in anuran larvae (Van Buskirk 2001, Relyea 2002a, Schoeppner and Relyea 2008). I removed 100 mL of water from each no-predator treatment and added 100 mL of aged tap water to equalize disturbance among experimental units. The cues were added daily to the experimental tubs 24 hours after predators had consumed tadpoles. It has been shown that predator cues aged for 24 – 96 hours retained their ability to induce behavioral responses in prey (Turner and Montgomery 2003, Peacor 2006).

The virus treatment was applied after 8 days of predator treatment application to allow adequate time for possible immune suppression associated with predatory stress. This duration was reasonable considering that morphological and behavioral responses from predator cues

have been observed as early as 6 days following exposure (Relyea 2003). Also, suppression of the hypothalamo-pituitary-adrenal axis has been documented in tadpoles within hours of exposure to alarm pheromones (Fraker et al. 2009). For the no-virus treatments, I added 411 μL of virus free media (Eagle's Minimal Essential Media [MEM]) to the tubs. The virus treatments received 411 μL of MEM containing ranavirus, which resulted in a final concentration of $10^3$ PFUs $mL^{-1}$. This concentration is within the range of doses used in other studies ($10^2$-$10^6$ PFUs $mL^{-1}$; Bollinger et al. 1999, Brunner et al. 2005, Pearman and Garner 2005) and is an environmentally relevant concentration (Rojas et al. 2005, Schock et al. 2008). The exposure duration lasted 4 days, which has been shown to initiate infection in the species used in my study (Hoverman et al. 2010; J. Hoverman, unpublished data). After 4 days, the water was changed and virus was not added again.

Every 2 days, I fed tadpoles ground TetraMin® at a ration of 6% of their body mass. A 6% ration of TetraMin® has been shown to be sufficient for normal growth and development and minimizes competitor-induced stress (Relyea 2002c). The food ration was calculated from 10 tadpoles that were independent of the experiment but reared under identical conditions as the control treatment (i.e., without virus and predators). This approach avoided possible stress on experimental tadpoles associated with handling and weighing. Before each feeding, the non-experimental tadpoles were blotted dry on a paper towel and weighed. The average mass of the tadpoles was used to calculate the 6% food ration. During the experiment, I adjusted the food ration to compensate for mortality by calculating the rations needed according to how many tadpoles were present in each experimental unit.

Water in tadpole and predator tubs was changed every 4 days to maintain water quality. To reduce the likelihood of virus contamination during the water changes, no-virus treatments

were handled first followed by virus treatments. Also, I used new nets, changed gloves and rinsed all surfaces with 0.75% Nolvasan® (2% chlorhexidine diacetate; Fort Dodge Animal Health, Fort Dodge, Iowa, USA) for at least 1 minute to prevent cross contamination (Bryan et al. 2009). Predator cues were added after each water change.

**Data Collection and Tadpole Observations**

To quantify tadpole behavioral responses to the treatments, I observed tadpole activity every day during the experiment using scan sampling (Altmann 1974). After slowly approaching the tubs, I scanned each tub (<5 sec) and recorded the number of tadpoles that were active and the total number of tadpoles in the tub. Tadpoles were considered active if they were moving in the water column or displaying tail movement. Percent activity was calculated as the total number of active tadpoles divided by the number of total tadpoles present in the tub. Percent activity was measured 10 times per tub on each observation day, with tub observations separated by <5 minutes. The mean of the 10 observations was used as the response variable for each tub. Tubs were observed between 0800 – 1000 hrs every day to reduce possible variation associated with diel patterns in activity. After the activity observations ended, an external stimulus (i.e., tapping) was applied once to the bottom of each tub, and I recorded the number of responsive tadpoles. Percent responsive was calculated identically to percent activity. Given that exposure to ranaviral disease can result in behavioral changes (Gray et al. 2009), this external stimulus provided an additional assessment of tadpole behavior that may not be captured with activity observations.

I monitored tadpole survival daily in each experimental unit and dead individuals were removed. I terminated the experiment after 3 wks PE, which is sufficient duration to observe mortality following ranavirus exposure for the species used in my study (Hoverman et al. 2010;

J. Hoverman, unpublished data), and calculated percent survival for each experimental unit. All surviving individuals were euthanized, weighed, and growth calculated as the difference between mean mass at the beginning of the experiment (based on the initial sample of 10 tadpoles) and the mass of each individual. Growth was averaged across individuals within an experimental unit prior to analysis. Two tadpoles that survived to the end of the experiment were randomly selected and necropsied to estimate viral load. Sections of the pronephros (kidneys) and liver were removed, placed in a 1.5-mL microcentrifuge tube, and frozen at -80°C until processing. Gloves were changed and a different set of sterile instruments was used for necropsy between each individual to prevent cross-contamination. Individuals that metamorphosed prior to the end of the experiment were deemed survivors and removed from the experimental unit. Metamorphs were removed after approximately 20% tail resorption, because a pilot study that I performed indicated that drowning occurred after this point due to simultaneous gill resorption. Because metamorphs were not included for the full duration of the experiment, they were not used in the analysis of growth or viral load. Also, none of the *L. sylvaticus* tadpoles exposed to ranavirus survived to the end of the experiment, thus growth and viral load were not analyzed for this species. All animal husbandry and euthanasia procedures followed an approved University of Tennessee IACUC protocol (#1816). Collection of egg masses was approved by the Tennessee Wildlife Resources Agency (Scientific Collection Permit #1990).

**Molecular Analyses**

Viral load has been used as an index of susceptibility to iridoviruses (e.g., Goldberg et al. 2003, Inendino et al. 2005), and was estimated using real-time PCR (qPCR). I pooled the liver and kidney sample from a given individual and extracted genomic DNA (gDNA) using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). I used the Qubit™ fluorometer and

the Quant-iT$^{TM}$ dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA).

I used the TaqMan qPCR assay following the methods of Picco et al. (2007). For each sample, I combined 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 1.5 µL of each primer (rtMCP-F [5' – ACA CCA CCG CCC AAA AGT AC – 3'] and rtMCP-R [5' – CCG TTC ATG ATG CGG ATA ATG – 3']), and 1.5 µL of rtMCP-probe (5'-CCT CAT CGT TCT GGC CAT CAA CCA-3'). I added 0.25 µg of gDNA from each sample to standardize the total amount of gDNA added to the tubes. Because the volume containing this amount of gDNA varied depending on the gDNA concentration of the sample, I used the values from the fluorometer to calculate how much of the sample to add. I then added DNA grade water to the sample to bring the total volume to 30 µL. A SmartCycler® (Cepheid, Sunnyvale, California) thermal cycler was used for the qPCR. In each run of the qPCR, I included 4 controls, which were a ranavirus-negative tadpole sample, a negative DNA grade water sample, a ranavirus-positive tadpole sample, and a cultured virus sample. For each sample, I recorded the cycle number at which the sample crossed the fluorescent threshold level (i.e., CT value). I used a standard curve obtained from a qPCR conducted with a concentration gradient of ranavirus and the CT value of each sample to calculate viral load. Viral load was averaged within each experimental unit prior to analyses.

**Data Analyses**

To test the effects of treatments on tadpole behavior, I conducted repeated-measures analysis-of-variance (ANOVA) for each variable (Sokal and Rohlf 1995). Main effects were virus treatment, predator treatment, and time (8 or 16 – 21 days for pre- and post-exposure to virus, respectively). The response variables were percent activity and percent responsiveness

averaged among experimental units. The pre-exposure analysis included the first 8 days of the experiment and provided behavioral evidence of cue effectiveness. The post-exposure to virus analysis included the remaining days of the experiment. However, the data for the post-exposure analysis was truncated when mortality rates exceeded 20% to prevent an increase in sample variation associated with fewer individuals available for calculating activity and responsiveness proportions. Whenever an interaction of main effects occurred, analyses were separated by predator or virus levels. In several analyses, I detected significant time by treatment interactions. Inasmuch as the main-effect responses associated with predator and virus treatments were of greatest interest, I included results addressing time by treatment interactions in Appendix B.

Mean percent survival, growth, and viral load were measured at the end of the experiment hence no repeated time effect was included in the analyses. I used a two-way ANOVA to test for differences in predator and virus effects on growth. I used a one-way ANOVA to test for the differences in mean viral load among predator treatments; a virus effect was not included in the model because all unexposed tadpoles tested negative for ranavirus. For all tests, if a significant predator effect existed, I used Fisher's least significant differences test for post-hoc comparisons (Westfall et al. 1999). I tested if my data followed a normal distribution for all response variables using a Shapiro-Wilk test (Sokal and Rohlf 1995). Normality was met in all cases ($P >$ 0.11) except for percent survival. For *H. chrysoscelis, L. clamitans,* and *P. feriarum*, high survival of all controls resulted in a non-normal distribution; therefore, I used a Kruskall-Wallis test to determine whether percent survival differed among virus and predator treatments in these cases. Mean percent survival was not tested for *L. sylvaticus*, because all individuals in virus-exposed experimental units died, which resulted in no variation – an ANOVA requirement (Sokal and Rohlf 1995). These results were qualitatively interpreted. Also, given that no *L.*

*sylvaticus* tadpoles that were exposed to ranavirus survived until the end of the experiment, I could not calculate or analyze growth for the virus treatment. However, I used a one-way ANOVA to test for differences in mean growth for this species among predator treatments for the no-exposure treatment. Data for one-way ANOVAs were normal ($P > 0.24$). All analyses were performed with SPSS 16.0 at $\alpha = 0.05$.

**RESULTS**

Prior to virus exposure, *Anax* and *Belostoma* cues reduced tadpole activity by 4 – 18% and 5 – 10%, respectively, compared to the controls (Table A.1, Figure A.1a). Activity was lower for tadpoles exposed to *Anax* cues compared to *Belostoma* cues for *H. chrysoscelis* and *P. feriarum* (Table A.1, Figure A.1a). Thus, the predator cues in my study reduced tadpole activity. There was no effect of the predator treatments on responsiveness except for *L. clamitans* (Table A.1, Figure A.1b), indicating that 3 of the 4 tadpole species responded similarly to an external stimulus regardless of the predator treatment. Mean responsiveness was 2% lower for *L. clamitans* tadpoles exposed to *Anax* cues (Table A.1, Figure A.1b). Time interacted with the predator effect for some species (Table A.1); these results are presented in Appendix B.

Following virus exposure, tadpole activity was 4 – 24% lower in cue-exposed treatments compared to the control (Table A.2, Figure A.2a). Activity levels continued to be lower for *P. feriarum* tadpoles exposed to *Anax* cues compared to *Belostoma* cues (Table A.2, Figure A.2a). Percent activity for *H. chrysoscelis* tadpoles was on average 3% lower in the virus-exposed treatment compared to the no-exposure treatment. The opposite relationship existed for *L. clamitans* tadpoles, where mean percent activity was 2% higher in the virus-exposed treatment. Percent responsiveness was 2 – 4% lower for *H. chrysoscelis* and *P. feriarum* tadpoles exposed to the virus (Table A.2, Figure A.2b). No additional significant differences were found for

predator and virus treatments, and these effects did not interact with each other (Table A.2, Figure A.2). Time interacted with the predator and virus effect for some species (Table A.2); these results are presented in Appendix B.

Virus exposure significantly reduced survival by 17 – 100%, with survival lowest in *L. clamitans* and *L. sylvaticus* tadpoles (Table A.3, Figure A.3). Virus exposure increased growth by 8% in surviving *L. clamitans* tadpoles (Table A.3, Figure A.3). No additional differences in mean survival, growth or viral load were detected between predator or virus treatments, and these effects did not interact (Table A.3, Figure A.3).

**DISCUSSION**

Consistent with previous amphibian research, predator cues from *Belostoma* and *Anax* significantly reduced activity of all tadpole species (Relyea 2001a). Across all species, the greatest reduction in activity from predator cues occurred in *H. chrysoscelis* (12 – 24%) and *L. sylvaticus* (10 – 14%) tadpoles. Both species tend to breed in temporary or semi-permanent wetlands with relatively few aquatic predators and developmental constraints to metamorphose before pond drying (Wellborn et al. 1996). Consequently, they typically display high activity levels in the absence of predators but respond strongly to the presence of predators (Relyea 2001a, Van Buskirk 2002). While *L. clamitans* also displayed reduced activity with predators, the magnitude of the response was lower compared to *L. sylvaticus* and *H. chrysoscelis*. Low activity for *L. clamitans* has been reported (Theimann and Wassersug 2000, Relyea 2001a), and usually is attributed to their association with permanent wetlands that contain more predators (Werner and McPeek 1994). Interestingly, *P. feriarum* displayed the lowest activity level. This species typically breeds in ephemeral wetlands similar to *L. sylvaticus* and *H. chrysoscelis* where high activity levels are needed to facilitate developmental rates. Previous research on the closely

related western chorus frog (*P. triseriata*) reported activity levels more than twice the magnitude that I observed in my experiment (Skelly 1995). While more research is required with this chorus frog species to characterize its general activity levels, it was clear that they display anti-predator behaviors that are consistent with a multitude of larval anurans.

I found that *Anax* cues reduced activity more than *Belostoma* cues for *H. chrysoscelis* and *P. feriarum* before virus exposure and for *P. feriarum* after virus exposure. However, there was no difference between the *Anax* and *Belostoma* treatments for *L. sylvaticus and L. clamitans*. Previous research has demonstrated that *Anax* larvae are more lethal than *Belostoma* and, consequently, tadpoles generally reduce activity level to a greater degree with more lethal predators (Relyea 2001b). While I did not find a consistent reduction in activity level with predation risk across the tested species, research in a diversity of systems has shown substantial variation in predator-induced plasticity in response to a common predator across species as well as within species across populations (Dodson 1988, Kohler and McPeek 1989, Werner 1991, Azevedo-Ramos et al. 1992, Spitze 1992, Peckarsky 1996, Relyea 2001a). Regardless of the mechanism driving the differences in the relative magnitude of tadpole responses to the predators, it was clear that the risk of predation was reducing tadpole activity level.

I found that before and after virus exposure, predator cues had very little effect on tadpole responsiveness, which was the external stimulus of tapping on the container. In general, most tadpoles (>70%) responded to this stimulus, despite reducing swimming activity in the presence of aquatic insect predators. I found that exposure to ranavirus reduced responsiveness by 2 – 4% in *P. feriarum* and *H. chrysoscelis*. Lethargy has been described as a gross sign associated with ranavirus infection (Gray et al. 2009), which may affect the detection rate of infected tadpoles by movement-stimulated predators (Lawler 1989). Alternatively, if tadpole responsiveness to an

attack was reduced due to ranavirus infection, capture probability by the predator may increase. Parris et al. (2004) found that ranavirus infected salamanders were depredated less than uninfected individuals; however, others have found that tadpoles infected with pathogens were predated more often than uninfected individuals (Lefcort and Blaustein 1995, Pfennig et al. 1999). More research is needed examining the effectiveness of tadpoles infected with a pathogen responding to and escaping predation attempts.

Percent activity for *H. chrysoscelis* tadpoles was 3% lower in the virus-exposed treatment compared to the control; however, the opposite relationship existed for *L. clamitans* tadpoles. Parris et al. (2004) reported that ranavirus-infected *A. tigrinum* that were not exposed to *Anax* predators had greater activity levels than uninfected individuals. They surmised that the increased activity might be pathogen induced and help facilitate transmission by increasing the likelihood of contact between hosts (Parris et al. 2004). Although I did not detect a statistical difference, *L. sylvaticus* that were exposed to ranavirus also exhibited greater activity in the no-predator treatment. Thus, the ranid tadpoles in my experiment appeared to respond similarly to ranavirus exposure as *A. tigrinum* (Parris et al. 2004). The lower activity of ranavirus-exposed *H. chrysoscelis* tadpoles may be related to the pathological responses associated with infection. Severe edema was noted in infected *H. chrysoscelis* tadpoles (N. Haislip, *unpublished data*), which likely reduced their mobility. Alternatively, it is possible that reduced movement was a host response to decrease the likelihood of infection. Several studies have shown that anuran larvae can recognize pathogens present in the aquatic environment and reduce activity to presumably decrease encounter rate with the pathogen (Lefcort and Blaustein 1995, Kiesecker et al. 1999, Theimann and Wassersug 2000). More research is needed to identify the mechanisms associated with reduced or increased activity of amphibian larvae exposed to pathogens.

Mortality from ranavirus occurred in all tadpole species in my experiment, providing additional evidence that ranaviruses infect multiple amphibian hosts (Duffus et al. 2008, Hoverman et al. 2010). Mortality was substantially higher for ranid tadpoles (*L. clamitans* = 62%, *L. sylvaticus* = 100%) than for hylid tadpoles (*H. chrysoscelis* = 17%, *P. feriarum* = 19%), which corresponds with die-off trends in wild populations (Green et al. 2002). However, ongoing research at the University of Tennessee indicates that mortality rates vary greatly across ranid and hylid species (J. Hoverman, *unpublished data*). These results suggest that more research is needed to understand the mechanism impacting mortality rates across species.

Exposure to predator cues and ranavirus did not synergistically increase mortality rates as predicted. Given that exposure to predator cues before and after exposure to ranavirus reduced tadpole activity for all species, the procedures I followed for cue generation and exposure were effective. The levels of cue concentration used in my study (0.57 mg of consumed tadpole biomass $L^{-1}$) have been shown to cause behavioral and morphological responses in tadpoles and are considered ecologically relevant (Van Buskirk 2001, Relyea 2002a, Schoeppner and Relyea 2008). Thus, it is reasonable to infer that exposure to *Anax* or *Belostoma* predators may not increase ranavirus-associated mortality for the tadpoles species used in my study. It is important to note that my study exposed tadpoles to predator cues for 8 days prior to ranavirus exposure, which may be insufficient time to cause immune suppression. Therefore, exposure to predator cues for longer durations or at higher concentrations may cause chronic stress and contribute to ranaviral disease emergence.

I found that virus exposure resulted in higher growth rates in surviving *L. clamitans* tadpoles. I documented that 19% of surviving *L. clamitans* had edema (N. Haislip, *unpublished data*), which could have increased growth estimates calculated from mass. However, if edema

was primarily responsible for driving this trend, I should have observed higher growth rates in *H. chrysoscelis* tadpoles, because this species experienced severe edema as well. Given these uncertainties and that higher growth rate was observed in only one species, more research is needed investigating the possible impacts of sublethal ranavirus infection on larval and post-metamorphic body size.

There were no differences in viral load among predator treatments, providing further support that cues from *Anax* or *Belostoma* predators may not function as significant ecological stressors for ranaviral disease. If this were true, I would have expected to observe higher viral loads (an index of virion density) in predator treatments. It is important to note that the relationship between viral load estimates from qPCR and ranaviral disease is unknown (Green et al. 2009); however in Chapter III, I found that viral load and mortality rates were correlated, which has been reported in other iridovirus studies (Inendino et al. 2005, Cotter et al. 2008).

**CONCLUSIONS**

There are very few studies that have examined the role of natural stressors in driving the susceptibility of amphibians to pathogens. In other animal taxa, natural stressors, including the threat of predation, have been shown to negatively affect immune parameters and, in some cases, increase susceptibility to diseases (Griffin 1989, Boonstra et al. 1998, Rigby and Jokela 2000). These findings have led to generalizations that natural stressors affect taxa similarly, hence contribute to host-pathogen dynamics (Carey et al. 1999). In Amphibia, it appears that the threat of predation may increase susceptibility of amphibians to certain pathogens (Theimann and Wassersug 2000, Parris et al. 2004, Belden and Kiesecker 2005), but this effect is not consistent among species or pathogens (Parris and Beaudoin 2004, Raffel et al. 2010). My study did not support the hypothesis that the threat of predation increases susceptibility to ranaviruses;

however, more studies are needed to verify if this trend holds true across additional amphibian taxa and viral types.

# CHAPTER III

## STAGE- AND SPECIES-SPECIFIC SUSCEPTIBILITY OF ANURANS TO RANAVIRUS

### INTRODUCTION

Pathogens are fundamental components of natural communities and have impacts that can vary from sequestration of resources from a host to large-scale population regulation (Anderson and May 1978, Price et al. 1986, Scott 1988, Sorensen and Minchella 1998). Moreover, by directly impacting the survival and reproduction of hosts, pathogens can affect community interactions and the structure and function of ecological communities (Holt 1977, Price et al. 1986, Scott 1988, Kiesecker and Blaustein 1999, Keesing et al. 2006, Lafferty et al. 2008). The recent emergence of pathogens in plant and animal communities has sparked interest in understanding the mechanisms driving host-pathogen dynamics (Daszak et al. 2000).

The role of pathogens in the recent declines of amphibians across the globe has received considerable attention (Goater and Ward 1992, Jancovich et al. 1997, Longcore et al. 1999, Kiesecker and Skelly 2001, Carey et al. 2003b, Wake and Vredenburg 2008). While amphibians are hosts for a diversity of pathogens (Wright and Whitaker 2000), many die-off events have been associated with infection by ranaviruses (Green et al. 2002, Carey et al. 2003a, Muths et al. 2006). Ranaviruses have been reported on 5 continents and are associated with nearly 50% of the reported amphibian mortality events in the United States (Green et al. 2002, Carey et al. 2003a, Carey et al. 2003b, Converse and Green 2005). In 96% of these reports, larvae and recently metamorphosed individuals experienced the greatest mortality. Although ranaviruses have been fairly well studied and characterized at the molecular level (Chinchar 2002, Chinchar et al. 2003, Chinchar et al. 2005, Williams et al. 2005), research has only recently begun to

examine the mechanisms associated with ranavirus emergence in wild populations (Gray et al. 2009).

The ability to combat pathogens in amphibians may be correlated with the development of the immune system. For amphibians that belong to the Order Anura, development is often categorized on a scale of 1 – 46 that was established by Gosner (1960), where stages 1 – 19, 20 – 25, 26 – 41, and 42 – 46 are embryos, hatchlings, larvae (i.e., tadpoles), and metamorphs, respectively. Previous studies suggest that there are varying degrees of immune system development across different amphibian life stages. Embryos and hatchlings (Gosner stages 1-24) lack many of the important components of the functional immune system because they have not yet fully developed organs such as pronephros (hereafter kidneys), liver, spleen, or thymus, and they do not produce T and B lymphocytes or major histocompatability complex (Fox 1963, Manning and Horton 1969, Du Pasquier et al. 1989, Hansen and Zapata 1998, Rollins-Smith 1998). Du Pasquier et al. (1989) found that the production of thymic lymphocytes increases during larval development, drops substantially at metamorphosis, and peaks in adult *Xenopus laevis*. Decreases in immune function during metamorphosis (Gosner stages 41-44) are probably related to endogenous production of glucocorticoids associated with restructuring organ systems for postmetamorphic life (Rollins-Smith 1998). Thus, peak immunity may occur between Gosner stages 30 – 40 for amphibian larvae.

The immunological changes that occur during anuran development should affect host-pathogen interactions (Rollins-Smith 1998, Gantress et al. 2003). Unfortunately, studies comparing the susceptibility of amphibians to pathogens at different developmental stages are rare. Adult *X. laevis* are able to overcome an infection from ranavirus, yet larvae are highly susceptible and experienced 80 – 100% mortality (Gantress et al. 2003). Scotthoefer et al.

(2003) infected *Lithobates pipiens* with trematodes at 3 development stages (Gosner stages 25-27, 31-39, and 42) and found that only those infected with trematodes at Gosner stage 25 experienced mortality. Embryos that were injected with ranavirus experienced 100% mortality (Tweedell and Granoff 1968). Collectively, these studies affirm that earlier developmental stages in anurans may be most susceptible to pathogens; however to date, no studies have tested all 4 larval developmental stages (embryo, hatchling, tadpole, and metamorph). Thus, the first objective of my research was to test for differences in susceptibility (as indexed by infection rates, mortality rates, and viral load) to ranavirus among developmental stages prior to the completion of metamorphosis.

Traditionally, disease ecology has focused on pathogens that attack a single host, which has limited our ecological understanding of disease dynamics driven by pathogens that infect multiple host species (Cleaveland et al. 2001, Dobson and Foufopoulos 2001, Parker and Gilbert 2004, Power and Mitchell 2004). While many amphibian pathogens including ranaviruses are capable of infecting multiple hosts, few studies have compared the relative susceptibility to pathogens among species. Schock et al. (2008) demonstrated that recently metamorphosed individuals of 4 amphibian species were differentially susceptible to different ranavirus strains. In addition, larval Cope's gray tree frogs (*Hyla chrysoscelis*) and pickerel frogs (*Lithobates palustris*) experience 3-fold greater mortality compared to eastern narrow-mouthed toads (*Gastrophryne carolinensis*) when exposed to ranaviruses (Hoverman et al. 2010). Field and laboratory studies have shown that *Anaxyrus americanus* tadpoles are more susceptible to the digenetic trematode, *Ribeiroia ondatrae*, than *H. versicolor* tadpoles (Johnson and Hartson 2009). Thus, differences in susceptibility to pathogens exist among amphibian species. There is a need to identify species that are highly susceptible to pathogens so that conservation initiatives

can be directed appropriately (Green et al. 2009). To date, very few studies have examined the relative susceptibility of amphibian larvae to ranaviruses (Schock et al. 2008, Hoverman et al. 2010). Moreover, these studies tested only one developmental stage, thus their results may be limited. The second objective of my study was to identify trends in the relative susceptibility to ranavirus for 7 North American anuran species, and to relate species-specific trends to life history and evolutionary characteristics of the hosts.

## METHODS

### Study Animals and Virus Isolate

I used 7 anuran species for my study: *L. clamitans, L. pipiens, L. sylvaticus, Pseudacris feriarum*, *H. chrysoscelis*, *Scaphiopus holbrookii,* and *A. americanus*, which are widely distributed in eastern North America (Lang et al. 2009). Between February – July 2009, I collected 7 – 20 egg masses for each species (except *H. chrysoscelis*, Table A.4). Egg masses were collected within 48 hours of deposition, rinsed with sterile water, and transported in 19-L buckets filled with aged tap water to the University of Tennessee Joe Johnson Animal Research and Teaching Unit (JARTU). For *H. chrysoscelis*, 9 amplexed breeding pairs were collected and transported to JARTU for oviposition in covered 11.7-L tub containing 7 L of aged tap water. Breeding pairs remained in containers <24 hrs before oviposition occurred.

Egg masses were placed outdoors the day after collection in 300-L wading pools filled with aged tap water to develop. These pools were covered with 60% shade cloth to prevent the colonization of aquatic insects or other amphibians. After hatching, tadpoles were maintained in these pools and fed rabbit chow (Purina, St. Louis, Missouri) and ground TetraMin® (Tetra, Blacksburg, Virginia) *ad libitum* until used in the experiments. The experiments began as individuals reached the appropriate developmental stages (see below). Prior to each

experimental trial, a subset of 10 initial individuals of the developmental stage to be tested was euthanized and frozen at -80˚ C for confirmation that they were negative for ranavirus using real-time quantitative polymerase chain reaction (qPCR, see Molecular Analyses section); all pre-experiment individuals tested negative.

A single isolate of *Ranavirus* was used for all experiments. The University of Georgia Veterinary Diagnostic and Investigational Laboratory (VDIL) extracted this isolate from morbid *L. catesbeianus* juveniles. Preliminary molecular analyses suggest that the isolate is similar to the ranavirus *frog virus 3* (GenBank accession no. EF101698, Miller et al. 2007), and it has been shown to be highly virulent in anuran larvae (Hoverman et al. 2010). Titrated stock solutions of the isolate were sent overnight by the VDIL to the University of Tennessee for the experiments.

**Experimental Trials**

For each species, I conducted a 14-d experimental trial for each of 4 developmental stages: 1) embryo (stage 11), 2) hatchling (stage 21), 3) larval (stage 30), and 4) pro-metamorphosis (stage 41, Gosner 1960). Embryos were defined as eggs containing developing embryos. Although it has been shown that embryos are extremely susceptible to ranavirus when injected with the virus (Tweedell and Granoff 1968), exposing embryos while in the egg capsule is a more ecologically relevant transmission route. Experimental units for all trials were 1-L tubs filled with 0.5 L of aged tap water. The tubs were placed at a common shelf height in a completely randomized design at the JARTU laboratory facility. Tubs were set approximately12 cm apart to reduce the likelihood of contamination among experiment units. I randomly assigned a single individual to each tub. Treatments included a no-virus control and a virus exposure of $10^3$ plaque-forming units (PFUs) $mL^{-1}$ (Hoverman et al. 2010) Both treatments were replicated 20 times for a total of 40 experimental units per trial.

I inoculated the water (i.e., bath exposure) with 29.5 μL of Eagle's Minimal Essential Media (MEM) for the no-virus control tubs and 29.5 μL of MEM containing the virus for the virus tubs. The resulting virus concentration was $10^3$ PFUs $mL^{-1}$, which is within the range of doses used in other studies ($10^2 – 10^6$ PFUs $mL^{-1}$; Bollinger et al. 1999, Brunner et al. 2005, Pearman and Garner 2005) and environmentally relevant (Rojas et al. 2005, Schock et al. 2008). Given that some species in my study developed rapidly (e.g., *S. holbrookii*), I used a 3-day exposure in an attempt to target the intended developmental stage rather than a subsequent stage. For *S. holbrookii* and *L. sylvaticus*, exposure during the embryo stage was less than 3 days because the embryos hatched prior to the end of the 3-day exposure. After 3 days, individuals were removed from the containers, rinsed with sterile water, and placed into a new container with 500-mL of fresh aged tap water. For the remainder of the experiment, water was changed every 3 days to maintain water quality.

After each water change, individuals in the larval and metamorph experiments were fed ground TetraMin® at a daily rate of 8% body weight (Relyea 2002). I weighed a group of 10 non-experimental individuals housed under identical conditions to calculate food rations, because weighing individuals in the experimental units would have increased the chance of contamination. The non-experimental individuals were weighed every 3 days prior to the water changes to estimate average mass and calculate the food ration for the treatment animals. Individuals in embryo and hatchling experiments were fed if they reached Gosner stage 25 prior to the end of the experiment, which is when yolk reserves are exhausted and jaw development is complete in most species (Thibaudeau and Altig 1999). After the initial exposure and water change, platforms were placed in the metamorph experimental units to allow individuals to crawl out of the water following gill resportion. Once individuals in the metamorph stage experiments

began tail resorption, water depth was slowly reduced until a minimal amount of water remained to provide moisture for the individual and TetraMin® was no longer added. Water was lowered because pilot studies revealed mortality of metamorphs associated with drowning upon tail resorption, even with floating platforms present. These individuals were fed 10 seed weevils (*Callosobruchus sp.*) every 3 days for the remainder of the experiment.

The experimental units were monitored 3 times daily for mortality and signs of disease. I noted any gross signs of ranaviral infections including loss of pigmentation, epithelial sloughing, edema, hemorrhaging, skin lesions, and erythema (Tweedell and Granoff 1968, Wolf et al. 1968, Gantress et al. 2003, Docherty et al. 2003). I also noted if individuals exhibited lethargy, inappetance, or loss of righting reflex (Jancovich et al. 1997, Bollinger et al. 1999, Docherty et al 2003). If an individual died during an experiment, Gosner stage was recorded and mass measured. For larvae and metamorphs, individuals were necropsied using sterilized forceps and scissors. Because the kidneys and liver are known sites of ranavirus infection (Gray et al. 2009), I removed sections of these organs from each individual, placed the pooled sample in a 1.5-mL microcentrifuge tube, and froze at -80°C for molecular testing. Embryos and hatchlings were rinsed with sterile water and frozen at -80˚C, because their small size prevented consistent necropsies. After 14 days, all live individuals were euthanized in benzocaine hydrochloride (1 g $L^{-1}$) and the identical necropsy procedures followed. I set 14 days as the experiment duration because previous research has shown this is sufficient duration to observe disease from ranavirus infection with a 3-day exposure (Hoverman et al. 2010). All animal husbandry and euthanasia procedures followed an approved University of Tennessee IACUC protocol (#1816). Collection of egg masses was approved by the Tennessee Wildlife Resources Agency (Scientific Collection Permit #1990).

**Molecular Analyses**

All experimental units exposed to ranavirus were tested using qPCR to estimate infection rate and viral load. Three random controls, as well as any controls that died, were also tested to confirm the absence of ranavirus; all controls were negative. Genomic DNA (gDNA) was extracted from a homogenate of the kidney and liver for tadpoles and metamorphs and from entire embryos (including vitelline membrane and mucoidal capsules) and hatchlings using a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). I used the Qubit™ fluorometer and the Quant-iT™ dsDNA BR Assay Kit to quantify the concentration of genomic DNA in each sample (Invitrogen Corp., Carlsbad, CA, USA), which was used to quantify viral load using qPCR (Yuan et al. 2006). I used the TaqMan qPCR assay for quantification of viral load in the samples. The qPCR amplified a 70-bp region of the ranavirus major capsid protein. For each sample, I combined 12.5 µL of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 1.5 µL of each primer (rtMCP-F [5' – ACA CCA CCG CCC AAA AGT AC – 3'] and rtMCP-R [5' – CCG TTC ATG ATG CGG ATA ATG – 3']), and 1.5 µL of rtMCP-probe (5'-CCT CAT CGT TCT GGC CAT CAA CCA-3'). I added 0.25 µg of gDNA from each sample to standardize the total amount of gDNA added to the tubes. Because the volume containing this amount of gDNA varied depending on the gDNA concentration of the sample, I used the values from the fluorometer to calculate how much of the sample to add. I then added DNA grade water to the sample to bring the total volume to 30 µL. A SmartCycler® (Cepheid, Sunnyvale, California) thermal cycler was used for the qPCR. In each run of the qPCR, I included 4 controls, which were a ranavirus-negative tadpole sample, a negative DNA grade water sample, a ranavirus-positive tadpole sample, and a cultured virus sample. For each sample, I recorded the cycle number at which the sample crossed the fluorescent threshold level,

which was set at 30 (i.e., CT value). I used a standard curve obtained from a qPCR conducted with a concentration gradient of ranavirus and the CT value of each sample to calculate viral load.

**Data Analyses**

The response variables for each experiment included mortality rate, infection rate, and viral load. For several of the experiments, there was mortality observed in the unexposed treatments. Given that these unexposed individuals tested negative for ranavirus infection, the mortality can be attributed to natural background mortality rather than contamination. In order to account for this mortality and facilitate unbiased comparisons of mortality rates among species and developmental stages, I divided the mortality rate of the virus-exposed treatment by the mortality rate of the unexposed treatment. Due to this standardization, I did not compare control and virus mortality rates.

Differences in infection and mortality rates were tested among species and developmental stages using logistic analysis (Stokes et al. 1999, Zar 1999). If the overall Wald's chi-square test was significant, I used binomial tests for 2 proportions that were Bonferroni corrected to test for pairwise differences (Zar 1999). I estimated the likelihood of infection and mortality for each treatment in comparison with the treatment having the lowest rate by calculating odds-ratio statistics (Stokes et al. 1999). If species and developmental stage effects interacted, I separated the analysis by species and performed a chi-square test for differences in mortality and infection among stages. I used an analysis-of-variance (ANOVA) to test for differences in viral load among species. Only individuals that were infected were included in the viral load analyses. Viral load was natural log transformed prior to analysis, because these data did not follow a normal distribution. If the ANOVA was significant, Tukey's Honestly Significant Difference

test was used for pairwise comparisons of viral load among species (Zar 1999). Lastly, I was interested in whether mortality rates were correlated with infection rates. Thus, mortality rates were regressed against infection rates for each experimental trial using simple linear regression (Zar 1999). All tests were performed at $\alpha = 0.05$ using the SAS® system (Littell et al. 1991, Stokes et al. 1999).

**RESULTS**

Across all species, mortality and infection rates for the hatchling, larval and metamorph stages were significantly greater than the embryo stage ($\chi^2_3 > 43.3$, $P < 0.001$; Figure A.4). In the hatchling, larval, and metamorph stages, the odds of mortality were 3X, 4X, and 5X greater, respectively, when exposed to ranavirus than the embryo stage. Across all developmental stages, mortality and infection rates were greatest for *L. sylvaticus* and *S. holbrookii*, and were lowest for *P. feriarum* and *A. americanus* ($\chi^2_6 > 40.67$, $P < 0.001$; Figure A.4). Intermediate mortality and infection occurred for *L. clamitans, L. pipiens,* and *H. chrysoscelis* (Figure A.4). Ranavirus exposed *L. sylvaticus* and *S. holbrookii* had 150X and 119X greater odds of mortality, respectively, than *P. feriarum*. There was a strong positive linear relationship between infection and mortality rates across species and developmental stages ($F_{1,20} = 74.5$, $P < 0.001$, $R^2 = 0.79$; Figure A.5). Also, viral load tended to be greatest for species with high mortality rates ($F_{6,24} = 5.7$, $P < 0.001$; Figure A.6).

Species and developmental stage effects interacted for mortality and infection rates ($\chi^2_{18} = 128.9$, $P < 0.001$); thus, logistic analyses were performed separately for each species. For all species except *L. sylvaticus*, mortality and infection rates differed among developmental stages ($\chi^2_3 > 12.6$, $P < 0.006$; Figure A.7). For *L. sylvaticus*, infection rates were high (>82%) and did not differ among stages ($\chi^2_3 = 6.3$, $P = 0.09$). Mortality and infection rates tended to be greatest

during the metamorph stage for all *Lithobates* species. Mortality also was greatest during the metamorph stage for *A. americanus*, but these individuals were not infected with ranavirus. Mortality and infection rates tended to be greatest during the larval stage for the two hylid species: *P. feriarum* and *H. chrysoscelis*. The greatest infection and mortality rates for *S. holbrookii* occurred during the embryo, hatchling and larval stages, and were lowest during metamorphosis (Figure A.7).

**DISCUSSION**

Embryos that were contained within eggs were the least susceptible stage across species when exposed to ranavirus in a water bath. Inasmuch as embryos do not have fully developed organs such as the mesonephros, liver, spleen, or thymus and they do not produce T and B lymphocytes or major histocompatability complex (Du Pasquier et al. 1989, Zettergren 2000), I expected that this stage would experience greater mortality than later developmental stages. Tweedell and Granoff (1968) demonstrated that *L. pipiens* embryos experienced high mortality (97 – 100%) within 3 – 12 days following injection with ranavirus. Thus, the vitelline membrane encasing the developing embryo or the mucopolysaccharide/mucoprotein capsules coating the surface of the egg afford protection against ranavirus infection. The mechanisms that contribute to this protection are unknown but may include structural barriers or anti-viral properties of the egg capsules or membrane. Amphibian egg membranes are known to be mechanical barriers to insecticides (Berrill et al. 1998, Pauli et al. 1999), which may inhibit intracellular movement of ranavirus virions. The vitelline membrane also may lack cell receptors necessary for virions to enter cells via receptor-mediated endocytosis (Chinchar 2002, Chinchar and Hyatt 2008), thereby thwarting infection. Finally, the mucopolysaccharide and mucoprotein capsules surrounding the embryo and forming the jelly substrate of fertilized egg masses may have antiviral properties.

Han et al. (2008) isolated a serine proteinase inhibitor from *Rana grahami* eggs that inhibited the growth of the bacterium *Bacillus subtilis*. No studies have tested whether this proteinase inhibitor exists in eggs of other amphibian species or whether it can inactivate ranavirus. Infection occurred in the embryo experiments for *S. holbrookii* and *L. sylvaticus*; however, embryos of these species hatched prior to the end of the 3-day virus challenge, hence exposing the hatchling to virions. No infection occurred during the embryo experiments in species that hatched after the virus challenge and first water change. Thus, it appears that eggs protect their developing embryos from ranavirus infection, but more research is needed.

I documented high mortality during metamorphosis for all species of *Lithobates* tested, which is frequently the stage associated with anuran die-offs in the wild (Green and Converse 2005, Greer et al. 2005). Cullen et al. (1995) and Cullen and Owens (2002) reported high susceptibility of several species of recently metamorphosed anurans compared to larvae or adults when exposed to ranavirus. High infection and mortality during metamorphosis may be associated with decreased immune function from endogenous production of corticosteroids and lymphocyte apoptosis (Flajnik et al. 1987; Rollins-Smith 1998, 2001), which has been demonstrated in *Xenopus laevis* (Rollins-Smith et al. 1993, Grant et al. 1998). All other species that I tested had low mortality and infection during metamorphosis. If immune function of these species resembled *X. laevis* then these species should have experienced high susceptibility as well to ranavirus during metamorphosis (Flajnik et al. 1987, Rollins-Smith 1998). Although I did not measure specific products of immune function (e.g., antibody production, leukocyte profiles; Rollins-Smith 2001, Davis et al. 2008), it appears that the model of *X. laevis* immunity during development may be inconsistent among anuran species based on my indices of susceptibility to ranavirus.

The greatest infection and mortality occurred during the hatchling stage for *S. holbrookii*, which was a different trend among the species that I tested. Infection and mortality rates decreased during the larval and metamorph stages, suggesting that immune function increased through development for this species. Compromised immunity during early development may be a consequence of physiological trade-offs associated with rapid development in this species. Spadefoots are among the fastest developing anuran species due to their association with ephemeral breeding sites (Newman 1992, Denver 1997). Zettergren (2000) reported cells synthesizing immunoglobulins (Ig) during embryogenesis and B lymphocytes circulating in pre-metamorphic *L. pipiens* at the onset of feeding. Leukocyte mobilization and anti-FV3 IgY antibody production have been reported as immune responses to ranavirus infection in *X. laevis* (Maniero et al. 2006, Morales et al. 2010). I hypothesize that development of these components of the amphibian immune system is delayed in *S. holbrookii* due to rapid growth during the embryo and hatchling stages.

Among species, *L. sylvaticus* was the most susceptible, with infection and mortality rates exceeding 80% in the hatchling, larval, and metamorph stages. High infection and mortality rates with this species have been reported in the wild across its geographic range in North America (e.g., Greer et al. 2005, Harp and Petranka 2006, Gahl and Calhoun 2010, Schock et al. 2010). Ongoing research at the University of Tennessee tested the relative susceptibility of tadpoles for 14 anuran and 5 urodelean species and found that *L. sylvaticus* was the most susceptible to ranavirus exposure (J. Hoverman and N. Haislip, *unpublished data*). Hoverman (*unpublished data*) found that *L. sylvaticus* died as quickly as 3 days following exposure to ranavirus, providing circumstantial evidence of poor innate immune response. Cotter et al. (2008) reported that poor lymphocyte production in the spleen was a mechanism driving high

susceptibility of larval *Ambystoma mexicanum* to the ranavirus, *Ambysoma tigrinum virus*. Significant increases in total leukocytes and natural killer cells are detected after 1 and 3 days post-infection with ranavirus, respectively, in *X. laevis* (Morales et al. 2010). Antibody production in pre-metamorphic *L. catesbeianus* and *X. laevis* has been reported (Haimovich and Du Pasquier 1973, Hsu and Du Pasquier 1984), and consequently these species are known to be relatively resistant to ranavirus infection (Robert et al. 2007; J. Hoverman, *unpublished data*). Thus, slow or minimal innate and adaptive immune response to ranavirus infection may be mechanisms contributing to high infection and mortality rates in ranavirus-exposed *L. sylvaticus*.

My results provide additional evidence that differences in susceptibility to ranavirus infection and disease exist among anuran species (Schock et al. 2008; J. Hoverman, *unpublished data*). In my study, species in Ranidae and Scaphiopodidae were most susceptible; very little mortality was observed for species in Hylidae and Bufonidae. It is possible that these differences in susceptibility are related to differences in life history and evolutionary characteristics of the hosts. Given that ranavirus transmission is facilitated by water, species inhabiting more permanent breeding sites may have evolved greater resistance to the virus due to high contact rates. Hoverman (*unpublished data*) provided some evidence for this trend, with *L. catesbeianus* exhibiting low susceptibility. However, in my study, 2 ranid species that are known to breed in permanent wetlands (*L. pipiens* and *L. clamitans*; Lang et al. 2009) had relatively high mortality (>40%). The very high mortality of *L. sylvaticus* and *S. holbrookii* supports the co-evolution hypothesis, but *A. americanus* and *P. feriarum* also breed in ephemeral habitats yet had low mortality rates in my study. Lastly, *H. chrysoscelis* experienced moderate mortality, which is reasonable considering this species breeds in ephemeral and permanent wetlands. Thus, the hypothesis that susceptibility is related to life history is partially supported.

To understand the role of host-virus co-evolution, comparing phylogenetic drivers of host susceptibility may provide insight (Storfer et al. 2007). For example, species in Hylidae and Bufonidae are considered more recently evolved than Ranidae or Scaphiopodidae (Frost et al. 2006), thus innate and adaptive immune responses for larvae of the former anuran families may be more advanced. Indeed, the mechanisms driving species susceptibility to ranavirus should have immunogenetic origins and need to be investigated (Cotter et al. 2008).

I found that viral load was greatest for species that had the highest mortality rates. Cotter et al. (2008) reported an increase in viral load as ranavirus infection and disease progressed in pre-metamorphic *A. mexicanum*. Green et al. (2009) cautioned the use of viral load estimates using qPCR to infer ranaviral disease. Cotter et al. (2008) and my study provide some evidence that viral load and ranaviral disease may be correlated, which has been shown with other iridoviruses (Inendino et al. 2005). I also found a strong correlation ($R^2 = 0.79$, $P < 0.001$) between ranavirus infection and mortality rates, which has been documented in other lab studies (Brunner et al. 2005; J. Hoverman*, unpublished data*). Field surveillance of ranavirus prevalence also indicates a strong correlation with mortality given that nearly all individuals that experience mortality at die-off sites are infected (M. Gray and D. Miller, *unpublished data*). Thus, measuring infection rates may provide a reasonable estimate of the likelihood of mortality in the field or lab. Conversely, measuring mortality rates in designed ranavirus-challenge experiments should provide reasonable estimates of infection rates and disease, which are costly to quantify (Green et al. 2009).

## CONCLUSIONS

My results indicate that susceptibility to ranavirus differs among developmental stages and species. High mortality rates were documented in all developmental stages except for the embryo stage. The classic model of amphibian immune function during development, based on *X. laevis,* suggests that immune function increases through development then drops at metamorphosis (Rollins-Smith 2001), thus mortality associated with ranavirus infection should be lowest during the larval (i.e., tadpole) stages. This trend did not occur for any of the anuran species that I tested, which may indicate that immune responses of North American anurans may differ from those of *X. laevis.*

My study is the first to report mortality of anuran hatchlings by ranavirus, which was greatest for *S. holbrookii*. The possibility for hatchling mortality from ranaviruses raises a significant conservation concern considering that detecting die-offs of hatchlings is extremely difficult in the wild. Differential susceptibility among developmental stages also indicates that studies that focus on one stage (e.g., Schock et al. 2008) may provide limited insight into species susceptibility. If testing only one stage is feasible, I recommend using the larval stage because mortality and infection rates were either greater or similar to hatchling and metamorph stages for most species.

If exposure to ranavirus during one developmental stage does not elicit an adaptive immune response that creates antibody memory (Hemingway et al. 2009), the probability of survival from embryo through metamorphosis is the product of the survival rate for each stage as per the Law of Independence (Allen 2006). If this is true, the probability of survival when exposed to ranavirus during all developmental stages equals 12% across all species tested in my study. Species of greatest risk include *L. sylvaticus* = 0%, *S. holbrookii* = 0.2%, *L. clamitans* = 3.6%, and *L. pipiens* = 4.6% survival. It is important to note that the FV3-like ranavirus that I

used in my study (Miller et al. 2007) is more virulent than FV3 (J. Hoverman, *unpublished data*). Nonetheless, these results emphasize the threat of ranavirus epizootics in some amphibian communities. Amphibian communities composed of highly susceptible species may be at greatest risk, considering these species may amplify free-floating viral concentrations at breeding sites through accelerated virion shedding. Thus, I propose that highly susceptible species instead of highly susceptible individuals may initiate superspreading events and ranavirus epizootics in an amphibian community (cf. Lloyd-Smith et al. 2005).

More research is needed investigating the role of immune function in regulating differences in susceptibility to ranavirus among anuran species. To date, only a handful of studies have quantified immune responses to ranavirus in pre-metamorphic amphibians (Gantress et al. 2003, Cotter et al. 2008). Identifying commonalities among immunogenetic, evolutionary and life history traits of susceptible species will improve our understanding of host-pathogen interactions (Richmond et al. 2009), and help facilitate identification of amphibian communities at greatest risk of ranavirus epizootics. To this end, I recommend that additional amphibian species and ranavirus strains be tested for relative susceptibility. Various multivariate techniques exist (e.g., canonical correspondence analysis, ter Braak 1986) that can elucidate patterns between host characteristics and indices of susceptibility. I also encourage studies that challenge amphibian species with ranavirus at each stage of development and follow individual survival through metamorphosis to test my hypothesis of stage independence to ranavirus susceptibility and my overall survival predictions. This knowledge is fundamental to developing stage-structured disease models that predict epizootic outcomes (Allen 2006).

## LITERATURE CITED

Allen, L. J. S. 2006. An introduction to mathematical biology. Prentice Hall, Upper Saddle River, New Jersey, USA.

Altmann, J. 1974. Observational study of behavior: Sampling methods. Behaviour 49:227-267.

Anderson, R. M. and R. M. May. 1978. Regulation and stability of host-parasite population interactions. I. Regulatory processes. Journal of Animal Ecology 47:219-247.

Ariel, E., J. Kielgast, H. E. Svart, K. Larsen, H. Tapiovaara, B. Bang Jensen, and R. Holopainen. 2009. Ranavirus in wild edible frogs *Pelophylax* kl. *esculentus* in Denmark. Diseases of Aquatic Organisms 85:7-14.

Azevedo-Ramos, C. M. Van Sluys, J. Hero, and W. E. Magnusson. 1992. Influence of tadpole movement on predation by odonate naiads. Journal of Herpetology 26:335-338.

Belden, L. K., and J. M. Kiesecker. 2005. Glucocorticosteroid hormone treatment of larval treefrogs increases infection by *Alaria sp* trematode cercariae. Journal of Parasitology 91:686-688.

Berrill, M. D. R. Coulson, L. McGillivray, B. D. Pauli. 1998. Toxicity of endosulfan to aquatic stages of anuran amphibians. Environmental Toxicology and Chemistry 17:1738-1744.

Bollinger, T. K., J. Mao, D. Schock, R. M. Brigham, and V. G. Chinchar. 1999. Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. Journal of Wildlife Diseases 35:413-429.

Boonstra, R., D. Hik, G. R. Singleton, A. Tinnikov. 1998. The impact of predator-induced stress on the snowshoe hare cycle. Ecological Monographs 68(3):371-394.

Brunner, J. L., D. M. Schock, and J. P. Collins. 2007. Transmission dynamics of the amphibian ranavirus *Ambystoma tigrinum* virus. Diseases of Aquatic Organisms 77:87-95.

Brunner, J. L., K. Richards, and J. P. Collins. 2005. Dose and host characteristics influence virulence of *ranavirus* infections. Oecologia 144:399-406.

Bryan, L. K., C. A. Baldwin, M. J. Gray, and D. L. Miller. 2009. Efficacy of select disinfectants at inactivating *Ranavirus*. Diseases of Aquatic Organisms 84:89-94.

Carey, C. 2000. Infectious disease and worldwide declines of amphibian populations, with comments on emerging diseases in coral reef organisms and in humans. Environmental Health Perspectives 108:143-150.

Carey, C. N. Cohen, L. Rollins-Smith. 1999. Amphibian declines: an immunological perspective. Developmental and Comparative Immunology 23:4509-472.

Carey, C., A. P. Pessier, and A. D. Peace. 2003a. Pathogens, infectious disease, and immune defenses. Pages 127-136 *in* R. D. Semlitsch, editor. Amphibian Conservation. Smithsonian Institute, Washington D. C., USA.

Carey, C., A. P. Pessier, and A. D. Peace. 2003b. Pathogens, infectious disease, and immune defenses. Pages 127-136 *in* R. D. Semlitsch, editor. Amphibian conservation. Smithsonian Institute, Washington D. C., USA.

Chinchar, V. G. 2002. Ranaviruses (family Iridoviridae): emerging cold-blooded killers - Brief review. Archives of Virology 147:447-470.

Chinchar, V. G. and A. D. Hyatt. 2008. Iridoviruses: general features. Pages 167-174 *in* Mahy, B. W. J., and M. H. V. Van Regenmortel, editor. Encylclopedia of virology, Vol. 3. Elsevier, Oxford, UK.

Chinchar, V. G., L. Bryan, J. Wang, S. Long, and G. D. Chinchar. 2003. Induction of apoptosis in *frog virus 3*-infected cells. Virology 306:303-312.

Chinchar, V. G., S. Essbauer, J. G. He, A. Hyatt, T. Miyazaki, V. Seligy, and T. Williams. 2005. Iridoviridae. Pages 163-175 *in* C. M. Faquet, M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball, editors. Virus taxonomy: 8th report of the international committee on the taxonomy of viruses. Elsevier, London, UK.

Cleaveland, S., M. K. Laurenson, and L. H. Taylor. 2001. Diseases of humans and their domestic mammals: pathogen characteristics, host range and the risk of emergence. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 356:991-999.

Collins, J. P. and M. L. Crump. 2009. Extinction in our times: global amphibian declines. Oxford University Press, New York, New York, USA.

Collins, J. P., and A. Storfer. 2003. Global amphibian declines: sorting the hypotheses. Diversity and Distributions 9:89-98.

Converse, A. K., and D. E. Green. 2005. Diseases of tadpoles. Pages 72-88 *in* S. K. Majumdar, J. E. Huffman, F. J. Brenner, and A. I. Panah, editor. Wildlife diseases: Landscape epidemiology, spatial distribution and utilization of remote sensing technology. The Pennsylvania Academy of Science, Easton, Pennsylvania, USA.

Cotter, J. D., A. Storfer, R. B. Page, C. K. Beachy, and S. R. Voss. 2008. Transcriptional response of Mexican axolotls to *Ambystoma tigrinum virus* (ATV) infection. BMC Genomics 9:493.

Cullen, B. R. and L. Owens. 2002. Experimental challenge and clinical cases of Bohle iridovirus (BIV) in native Australian anurans. Diseases of Aquatic Organisms 49:83-92.

Cullen, B. R., L. Owens, and R. J. Whittington. 1995. Experimental infection of Australian anurans (*Limnodynastes terraereginae* and *Litoria latopalmata*) with Bohle iridovirus. Diseases of Aquatic Organisms 23:83-92.

Cunningham, A. A., T. E. S. Langton, P. M. Bennett, S. E. N. Drury, R. E. Gough, and J. K. Kirkwood. 1993. Unusual mortality associated with poxvirus-like particles in frogs (*Rana temporaria*). Veterinary Record 133:141-142.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife: threats to biodiversity and human health. Science 287:443-449.

Daszak, P., L. Berger, A. A. Cunningham, A. D. Hyatt, D. E. Green, and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5(6):735-748.

Davis, A. K. and J. C. Maerz. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Functional Ecology 22:760-772.

Denver, R. J. 1997. Environmental stress as a developmental cue: corticotropin-releasing hormone is a proximate mediator of adaptive phenotypic plasticity in amphibian metamorphosis. Hormones and Behavior 31:169-179.

Dobson, A., and J. Foufopoulos. 2001. Emerging infectious pathogens of wildlife. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 356:1001-1012.

Docherty, D. E., C. U. Meteyer, J. Wang, J. Mao, S. T. Case, and V. G. Chinchar. 2003. Diagnostic and molecular evaluation of three iridovirus-associated salamander mortality events. Journal of Wildlife Diseases 39:556-566.

Dodson, S. 1988. The ecological role of chemical stimuli for the zooplankton – Predator-avoidance behavior in *Daphnia*. Limnology and Oceanography 33(6):1431-1439.

Drury, S. E., R. E. Gough, and A. A. Cunningham. 1995. Isolation of an iridovirus-like agent from common frogs (*Rana temporaria*). Veterinary Record 137:72-73.

Du Pasquier, L. and N. Weiss. 1973. The thymus during the ontogeny of the toad *Xenopus laevis*: growth, membrane-bound immunoglobulins, and mixed lymphocyte reaction. European Journal of Immunology 3:773-777.

Du Pasquier, L., J. Schwager, and M. F. Flajnik. 1989. The immune system of *Xenopus*. Annual Review of Immunology 7:251-275.

Ducoroy, P., M. Lesourd, M. R. Padros, and A. Tournefier. 1999. Natural and induced apoptosis during lymphocyte development in the axolotl. Developmental and Comparative Immunology 23:241-252.

Duffus A. L. J., B. D. Pauli, K. Wozney, C. R. Brunetti, and M. Berrill. 2008. *Frog virus 3*-like infections in aquatic amphibian communities. Journal of Wildlife Diseases 44:109-120.

Flajnik, M. F., E. Hsu, J. F. Kaufman, and L. Du Pasquier. 1987. Changes in the immune system during metamorphosis of *Xenopus.* Immunology Today 8:58-64.

Fowler, M. and R. Miller. 2007. Zoo and wild animal medicine current therapy. Saunders, Philadelphia, USA.

Fox, H. 1963. The amphibian pronephros. Quarterly Review of Biology 38:1-25.

Fraker, M. E. 2008. The dynamics of predation risk assessment: responses of anuran larvae to chemical cues of predators. Journal of Animal Ecology 77:638-645.

Fraker, M. E., F. Hu, V. Cuddapah, S. A. McCollum, R. A. Relyea, J. Hempel, and R. J. Denver. 2009. Characterization of an alarm pheromone secreted by amphibian tadpoles that induces behavioral inhibition and suppression of the neuroendocrine stress axis. Hormones and Behavior 55:520-529.

Frost, D. R., T. Grant, J. Faivovich, R. H. Bain, A. Haas, C. F. B. Haddad, R. O. De Sa, A. Channing, M. Wilkinson, S. C. Donnellan, C. J. Raxworthy, P. Moler, R. C. Drewes, R.

A. Nussbaum, J. D. Lynch, D. M. Green, and W. C. Wheeler. 2006. The amphibian tree of life. Bulletin of the American museum of natural history 297:1-370.

Gahl, M. K. and A. J. K. Calhoun. 2010. The role of multiple stressors in ranavirus-caused amphibian mortalities in Acadia national park wetlands. Canadian Journal of Zoology 88:108-121.

Gantress, J., G. D. Maniero, N. Cohen, and J. Robert. 2003. Development and characterization of a model system to study amphibian immune responses to iridoviruses. Virology 311:254-262.

Goater, C. P., and P. I. Ward. 1992. Negative effects of *Rhabdias-bufonis* (nematode) on the growth and survival of toads (*Bufo bufo)*. Oecologia 89:161-165.

Goldberg, T. L., D. A. Coleman, E. C. Grant, K. R. Inendino, and D. P. Philipp. 2003. Strain variation in an emerging iridovirus of warm-water fishes. Journal of Virology 77(16):8812-8818.

Gosner, K. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183-190.

Granoff, A., P. E. Came, and K. A. Raffert. 1965. The isolation and properties of viruses from *Rana pipiens*: their possible relationship to the renal adenocarcinoma of the leopard frog. Annals New York Academy of Sciences 126:237-255.

Grant, P., R. H. Clothier, R. O. Johnson, and L. N. Ruben. 1998. In situ lymphocyte apoptosis in larval *Xenopus laevis*, the South African clawed toad. Developmental and Comparative Immunology 22(4):449-455.

Gray, M. J., D. L. Miller, and J. T. Hoverman. 2009. Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87:243-266.

Green, D. E. and K. A. Converse. 2005. Diseases of frogs and toads. Pages 89-117 *in* S. K. Majumdar, J. E. Huffman, F. J. Brenner, and A. I. Panah, editors. Wildlife diseases: landscape epidemiology, spatial distribution and utilization of remote sensing technology. Pennsylvania Academy of Science, Easton, Pennsylvania, USA.

Green, D. E., K. A. Converse, and A. K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Annals of the New York Academy of Sciences 969:323-339.

Green, D. E., M. J. Gray, and D. L. Miller. 2009. Disease monitoring and biosecurity. Pages 481-50 *in* Dodd C. K. Jr. Amphibian ecology and conservation. Oxford University Press, New York, New York.

Greer, A. L., C. J. Briggs, and J. P Collins. 2008. Testing a key assumption of host-pathogen theory: density and disease transmission. Oikos 117:1667-1673.

Greer, A. L., M. Berrill, and P. J. Wilson. 2005. Five amphibian mortality events associated with ranavirus infection in south central Ontario, Canada. Diseases of Aquatic Organisms 67:9-14.

Greger, M. 2007. The human/animal interface: Emergence and resurgence of zoonotic infectious diseases. Critical Reviews in Microbiology 33:243-299.

Griffin, J. F. T. 1989. Stress and immunity: a unifying concept. Veterinary Immunology and Immunopathology 20:263-312.

Haimovich, J. and L. Du Pasquier. 1973. Specificity of antibodies in amphibian larvae possessing a small number of lymphocytes. Proceedings of the National Academy of Sciences 70(6):1898-1902.

Han, Y., H. Yu, X. Yang, H. H. Rees, J. Liu, and R. Lai. 2008. A serine proteinase inhibitor from frog eggs with bacteriostatic activity. Comparative Biochemistry and Physiology, Part B 149:58-62.

Hansen, J. D. and A. G. Zapata. 1998. Lymphocyte development in fish and amphibians. Immunological Reviews 166:199-220.

Harp, E. M. and J. W. Petranka. 2006. Ranavirus in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. Journal of Wildlife Diseases 42(2): 307-318.

Hemingway, V., J. Brunner, R. Speare, and L. Berger. 2009. Viral and bacterial diseases of amphibians. Pages 2963-2985 *in* H. Heatwole, editor. Amphibian Biology Vol. 8. Surrey Beatty and Sons, Baulkham Hills, Australia.

Holt, R. D. 1977. Predation, apparent competition, and structure of prey communities. Theoretical Population Biology 12:197-229.

Hoverman, J. T., M. J. Gray, and D. L. Miller. 2010. Anuran susceptibility to ranaviruses: role of species identity, exposure route, and a novel virus isolate. Diseases of Aquatic Organisms 89:97-107.

Hsu, E. and L Du Pasquier. 1984. Ontogeny of the immune system in *Xenopus*. II. Antibody repertoire differences between larvae and adults. Differentiation 28:116-122.

Inendino, K. R., E. C. Grant, D. P. Philipp, and T. L. Goldberg. 2005. Effects of factors related to water quality and population density on the sensitivity of juvenile largemouth bass to mortality induced by viral infection. Journal of Aquatic Animal Health 17:304-314.

Jancovich, J. K., E. W. Davidson, J. F. Morado, B. L. Jacobs, and J. P. Collins. 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Diseases of Aquatic Organisms 31:161-167.

Johnson, P. T. J. and R. B. Hartson. 2009. All hosts are not equal: explaining differential patterns of malformations in an amphibian community. Journal of Animal Ecology 78:191-201.

Keesing, F., R. D. Holt, and R. S. Ostfeld. 2006. Effects of species diversity on disease risk. Ecology Letters 9:485-498.

Kiesecker, J. M. and A. R. Blaustein. 1999. Pathogen reverses competition between larval amphibians. Ecology 80:2442-2448.

Kiesecker, J. M. and D. K. Skelly. 2001. Effects of disease and pond drying on gray tree frog growth, development, and survival. Ecology 82:1956-1963.

Kiesecker, J. M., D. K. Skelly, K. H. Beard, E. Preisser. 1999. Behavioral reduction of infection risk. Proceedings of the National Academy of Sciences of the United States of America 96(16):9165-9168.

Kohler, S. L. and M. A. McPeek. 1989. Predation risk and the foraging behavior of competing stream insects. Ecology 70(6):1811-1825.

Lafferty, K. D., S. Allesina, M. Arim, C. J. Briggs, G. De Leo, A. P. Dobson, J. A. Dunne, P. T. J. Johnson, A. M. Kuris, D. J. Marcogliese, N. D. Martinez, J. Memmott, P. A. Marquet, J. P. McLaughlin, E. A. Mordecai, M. Pascual, R. Poulin, and D. W. Thieltges. 2008. Parasites in food webs: the ultimate missing links. Ecology Letters 11:533-546.

Lang, E., C. Gerhardt, and C. Davidson. 2009. The frogs and toads of North America. Houghton Mifflin Company, New York, New York, USA.

Lawler, S. 1989. Behavioural responses to predators and predation risk in four species of larval anurans. Animal Behaviour 38:1039-1047.

Lefcort, H. and A. R. Blaustein. 1995. Disease, predator avoidance, and vulnerability to predation in tadpoles. Oikos 74(3):469-474.

Lefcort, H. and S. M. Eiger. 1993. Antipredatory behaviour of feverish tadpoles: implications for pathogen transmission. Behaviour 126(1-2):13-27.

Lima, S. L. and L. M. Dill. 1990. Behavioural decisions made under the risk of predation: a review and prospectus. Canadian Journal of Zoology-Revue Canadienne De Zoologie 68:619-640.

Littell, R. C., R. J. Freund, and P. C. Spector. 1991. SAS® system for linear models. SAS Institute, Cary, North Carolina, USA.

Lloyd-Smith J. O, S. J. Schreiber, P. E. Kopp, and W. M. Getz. 2005. Superspreading and the effect of individual variation of disease emergence. Nature 438:355-359.

Longcore, J. E., A. P. Pessier, and D. K. Nichols. 1999. *Batrachochytrium dendrobatidis* gen et sp nov, a chytrid pathogenic to amphibians. Mycologia 91:219-227.

Maniero, G., H. Morales, J. Gantress, and J. Robert. 2006. Generation of a long-lasting, protective, and neutralizing antibody response to the ranavirus *FV3* by the frog *Xenopus*. Developmental and Comparative Immunology 30:649-657.

Manning, M. J. and J. D. Horton. 1969. Histogenesis of lymphoid organs in larvae of South African clawed toad, *Xenopus laevis* (Daudin). Journal of Embryology and Experimental Morphology 22:265-277.

Martin, L. B. 2009. Stress and immunity in wild vertebrates: Timing is everything. General and Comparative Endocrinology 163:70-76.

Miller, D. L., S. Rajeev, M. J. Gray, and C. A. Baldwin. 2007. *Frog virus 3* infection, cultured American bullfrogs. Emerging Infectious Diseases 13:342-343.

Morales, H. D., L. Abramowitz, J. Gertz, J. Sowa, A. Vogel, and J. Robert. 2010. Immune responses and permissiveness to ranavirus infection of peritoneal leukocytes in the frog *Xenopus laevis*. Journal of Virology 84(10):4912-4922.

Morin, P. J. 1983. Predation, competition, and the composition of larval anuran guilds. Ecological Monographs 53(2):119-138.

Muths, E., A. L. Gallant, E. H. C. Campbell, W. A. Battaglin, D. E. Green, J. S. Staiger, S. C. Walls, M. S. Gunzburger, and R. F. Kearney. 2006. The Amphibian Research and Monitoring Initiative (ARMI): 5-year report: U.S. Geological Survey Scientific Investigations Report 2006.

Newman, R. A. 1992. Adaptive plasticity in amphibian metamorphosis. BioScience 42(9):671-678.

Pauli, B. D. D. R. Coulson, M. Berrill. 1999. Sensitivity of amphibian embryos and tadpoles to MIMIC 240LV insecticide following single or double exposures. Environmental Toxicology and Chemistry 18:2538-2544.

Parker, I. M. and G. S. Gilbert. 2004. The evolutionary ecology of novel plant-pathogen interactions. Annual Review of Ecology, Evolution, and Systematics **35**:675-700.

Parris, M. J., A. Davis, and J. P. Collins. 2004. Single-host pathogen effects on mortality and behavioral responses to predators in salamanders (Urodela: Ambystomatidae). Canadian Journal of Zoology 82:1477-1483.

Parris, M. J. and J. G. Beaudoin. 2004. Chytridiomycosis impacts predator-prey interactions in larval amphibian communities. Oecologia 140: 626-632.

Peacor, S. D. 2006. Behavioural response of bullfrog tadpoles to chemical cues of predation risk are affected by cue age and water source. Hydrobiologia 573:39-44.

Pearman, P. B. and T. W. J. Garner. 2005. Susceptibility of Italian agile frog populations to an emerging strain of *Ranavirus* parallels population genetic diversity. Ecology Letters 8:401-408.

Peckarsky, B. L. 1996. Alternative predator avoidance syndromes of stream-dwelling mayfly larvae. Ecology 77(6):1888-1905.

Pfennig, D. W., J. P. Collins, and R. E. Ziemba. 1999. A test of alternative hypotheses for kin recognition in cannibalistic tiger salamanders. Behavioral Ecology 10(4):436-443.

Picco, A. M., J. L. Brunner, and J. P. Collins. 2007. Susceptibility of the endangered California tiger salamander, *Ambystoma californiense*, to *Ranavirus* infection. Journal of Wildlife Diseases 43:286-290.

Power, A. G. and C. E. Mitchell. 2004. Pathogen spillover in disease epidemics. American Naturalist 164:S79-S89.

Price, P. W., M. Westoby, B. Rice, P. R. Atsatt, R. S. Fritz, J. N. Thompson, and K. Mobley. 1986. Parasite mediation in ecological interactions. Annual Review of Ecology and Systematics 17:487-505.

Raffel, T. R., J. T. Hoverman, N. T. Halstead, P. J. Michel, and J. R. Rohr. 2010. Parasitism in a community context: trait-mediated interactions with competition and predation. Ecology 91(7):1900-1907.

Relyea, R. A. 2001a. Morphological and behavioral plasticity of larval anurans in response to different predators. Ecology 82:523-540.

Relyea, R. A. 2001b. The relationship between predation risk and antipredator responses in larval anurans. Ecology 82:541-554.

Relyea, R. A. 2002a. Costs of phenotypic plasticity. The American Naturalist 159:272-282.

Relyea, R. A. 2002b. Local population differences in phenotypic plasticity: predator-induced changes in wood frog tadpoles. Ecological Monographs 72:77-93.

Relyea, R. A. 2002c. Competitor-induced plasticity in tadpoles: consequences, cues, and connections to predator-induced plasticity. Ecological Monographs 72:523-540.

Relyea, R. A. 2003. Predators come and predators go: The reversibility of predator-induced traits. Ecology 84:1840-1848.

Relyea, R. A. and J. R. Auld. 2005. Predator- and competitor-induced plasticity: How changes in foraging morphology affect phenotypic trade-offs. Ecology 86(7):1723-1729.

Relyea, R. A. and E. E. Werner. 1999. Quantifying the relation between predator-induced behavior and growth performance in larval anurans. Ecology 80:2117-2124.

Richmond, J. Q., A. E. Savage, K. R. Zamudio, and E. B. Rosenblum. 2009. Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity. BioScience 59(4):311-320

Rigby, M. C. and J. Jokela. 2000. Predator avoidance and immune defence: costs and trade-offs in snails. Proceeding of the Royal Society of London. 267:171-176.

Robert, J., L. Abramowitz, J. Gantress, and H. D. Morales. 2007. *Xenopus laevis*: A possible vector of ranavirus infection? Journal of Wildlife Diseases 43:645-652.

Rojas, S., K. Richards, J. K. Jancovich, and E. W. Davidson. 2005. Influence of temperature on *Ranavirus* infection in larval salamanders *Ambystoma tigrinum*. Diseases of Aquatic Organisms 63:95-100.

Rollins-Smith, L. A. 1998. Metamorphosis and the amphibian immune system. Immunological Reviews 166:221-230.

Rollins-Smith, L. A. 2001. Neurendocrine-immune system interactions in amphibians: Implications for understanding global amphibian declines. Immunologic Research 23(2/3):273-280.

Rollins-Smith, L. A. and P. J. Blair. 1993. The effects of corticosteroid hormones and thyroid hormones on lymphocyte viability and proliferation during development and metamorphosis of *Xenopus laevis*. Differentiation 54:155-160.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology and Evolution 19:249-255.

Schock, D. M. and T. K. Bollinger. 2005. An apparent decline of Northern leopard frogs (*Rana pipiens)* on the Rafferty dam mitigation lands near Estevan, Saskatchewan. Blue Jay 63:144-154.

Schock, D. M., G. R. Ruthig, J. P. Collins, S. J. Kutz, S. Carriere, R. J. Gau, A. M. Veitch, N. C. Larter, D. P. Tate, G. Guthrie, D. G. Allaire, and R. A. Popko. 2010. Amphibian chytrid fungus and ranaviruses in the Northwest territories, Canada. Diseases of Aquatic Organisms *in press.*

Schock, D. M., T. K. Bollinger, V. G. Chinchar, J. K. Jancovich, and J. P. Collins. 2008. Experimental evidence that amphibian ranaviruses are multi-host pathogens. Copeia 2008(1):133-143.

Schoeppner, N. M. and R. A. Relyea. 2005. Damage, digestion, and defence: the roles of alarm cues and kairomones for inducing prey defenses. Ecology Letters 8:505-512.

Schoeppner, N. M. and R. A. Relyea. 2008. Detecting small environmental differences: risk-response curves for predator-induced behavior and morphology. Oecologia 154:743-754.

Scott, M. E. 1988. The impact of infection and disease on animal populations: Implications for conservation biology. Conservation Biology 2(1):40-56.

Scotthoefer, A. M., R. A. Cole, and V. R. Beasley. 2003. Relationship of tadpole stage to location of echinostome cercariae encystment and the consequences for tadpole survival. Journal of Parasitology 89(3):475-482.

Skelly, D. K. 1995. A behaviorl trade-off and its consequences for the distribution of pseudacris treefrog larvae. Ecology 76(1):150-164.

Sokal, R. R. and F. J. Rohlf. 1995. Biometry: the principles and practice of statistics in biological research. W. H. Freeman & Co., New York, New York, USA.

Spitze, K. 1992. Predator-mediated plasticity of prey life-history and morphology: *Chaoborus americanus* predation on *Daphnia pulex*. American Naturalist 139:229-247.

Sorensen, R. E. and D. J. Minchella. 1998. Parasite influences on host life history: *Echinostoma revolutum* parasitism of *Lymnaea elodes* snails. Oecologia 115:188-195.

Stokes, M. E., C. S. Davis, and G. G. Kock. 1999. Categorical data analysis using the SAS® system. SAS Institute, Cary, North Carolina, USA.

Stoks, R., M. De Block, S. Slos, W. Van Doorslaer, and J. Rolff. 2006. Time constraints mediate predator-induced plasticity in immune function, condition, and life history. Ecology 87(4):809-815.

Storfer A., M. E. Alfaro, B. J. Ridenhour, J. K. Jancovich, S. G. Mech, M. J. Parris, and J. P. Collins. 2007. Phylogenetic concordance analysis shows an emerging pathogen is novel and endemic. Ecology Letters 10:1075-1083.

Stuart, S. N., J. S. Chanson, N. A. Cox, B. E. Young, A. S. L. Rodrigues, D. L. Fischman, and R. W. Waller. 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786.

Teacher, A. G. F., A. A. Cunningham, and T. W. J. Garner. 2010. Assessing the long-term impact of *Ranavirus* infection in wild common frog populations. Animal Conservation 13:514-522.

ter Braak, C. J. F. 1986. Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. Ecology 67(5):1167-1179.

Thibaudeau, G. and R. Altig. 1999. Chapter 7/Endotrophic anurans. Pages 170-188 *in* R. W. McDiarmid and R. Altig, editor. Tadpoles: the biology of anuran larvae. University of Chicago Press, Chicago, Illinois, USA.

Thiemann, G. W., and R. J. Wassersug. 2000. Patterns and consequences of behavioural responses to predators and parasites in *Rana* tadpoles. Biological Journal of the Linnean Society 71:513-528.

Tournefier, A. 1982. Corticosteroid action on lymphocyte subpopulations and humoral immune response of axolotl (urodele amphibian). Immunology 46:155-162.

Turner, A. M. and S. L. Montgomery. 2003. Spatial and temporal scales of predator avoidance: experiments with fish and snails. Ecology 84:616-622.

Tweedell, K. and A. Granoff. 1968. Viruses and renal carcinoma of *Rana pipiens*. V. Effect of *Frog Virus 3* on developing frog embryos and larvae. Journal of the National Cancer Institute 40:407-410.

Van Buskirk, J. 2001. Specific induced responses to different predator species in anuran larvae. Journal of Evolutionary Biology 14:482-489.

Van Buskirk, J. 2002. A comparative test of the adaptive plasticity hypothesis: relationships between habitat and phenotype in anuran larvae. American Naturalist 160(1):87-102.

Van Buskirk, J., S. A. McCollum, and E. E. Werner. 1997. Natural selection for environmentally induced phenotypes in tadpoles. Evolution 51(6):1983-1992.

Wake, D. B. and V. T. Vrendenburg. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105:11466-11473.

Wellborn, G. A., D. K. Skelly, and E. E. Werner. 1996. Creating community structure across a freshwater habitat gradient. Annual Review of Ecology and Systematics 27:337-363.

Werner, E. E. 1986. Amphibian metamorphosis: growth rate, predation risk, and the optimal size at transformation. The American Naturalist 128:319-341.

Werner, E. E. 1991. Nonlethal effects of a predator on competitive interactions between 2 anuran larvae. Ecology 72(5):1709-1720.

Werner, E. E. and B. R. Anholt. 1996. Predator-induced behavioral indirect effects: consequences to competitive interactions in anuran larvae. Ecology 77:157-169.

Werner, E. E. and M. A. McPeek. 1994. Direct and indirect effects of predators on two anuran species along an environmental gradient. Ecology 75(5):1368-1382.

Westfall, P. H., R. D. Tobias, D. Rom, R. D. Wolfinger, and Y. Hochberg. 1999. Multiple comparisons and multiple tests using the SAS® system. SAS Institute, Cary North Carolina, USA.

Whiles, M. R., K. R. Lips, C. M. Pringle, S. S. Kilham, R. J. Bixby, R. Brenes, S. Connelly, J. C. Colon-Gaud, M. Hunte-Brown, A. D. Huryn, C. Montgomery, and S. Peterson. 2006. The

effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Frontiers in Ecology and the Environment 4:27-34.

Wilbur, H. M. 1972. Competition, predation, and the structure of the Ambystoma-Rana sylvatica community. Ecology 53(1):3-21.

Williams, T., V. Barbosa-Solomieu, and G. D. Chinchar. 2005. A decade of advances in iridovirus research. Pages 173-248 *in* K. Maramorosch and A. Shatkin, editors. Advances in virus research. Volume 65. Academic Press, New York, New York, USA.

Wolf, K., G. L. Bullock, C. E. Dunbar, and M. C. Quimby. 1968. *Tadpole Edema Virus*: a viscerotropic pathogen for anuran amphibians. Journal of Infectious Diseases 118:253-262.

Wright, K. M. and B. R. Whitaker. 2001. Amphibian medicine and captive husbandry. Krieger Publishing Company, Malabar, Florida, USA.

Yuan, J. S., A. Reed, F. Chen and C. N. Stewart Jr. 2006. Statistical analysis of real-time PCR data. BMC Bioinformatics 7:85-97.

Zar, J. H. 1999. Biostatistical analysis, 4th edition. Prentice Hall, Upper Saddle River, New Jersey, USA.

Zettergren, L. D. 2000. Ontogeny of B cells expressing IgM in embryonic and larval tissues of the American grass frog, *Rana pipiens*. Journal of Experimental Zoology 286:737-744.

# APPENDICES

# APPENDIX A

# TABLES AND FIGURES

Table A.1. Results of repeated-measures ANOVAs testing the effects of predator cue on the behavioral responses of 4 tadpole species during 8 consecutive days prior to exposure to ranavirus.

| Behavior[1] | Effects[2] | *Hyla chrysoscelis* | | | *Pseudacris feriarum* | | | *Lithobates sylvaticus* | | | *Lithobates clamitans* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | df | *F*[3] | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Activity | Predator | 2,27 | 79.1 | <0.001 | 2,27 | 64.3 | <0.001 | 2,27 | 61.2 | <0.001 | 2,27 | 16.7 | <0.001 |
| | Time | 7,21 | 73.0 | <0.001 | 7,21 | 77.1 | <0.001 | 7,21 | 156.7 | <0.001 | 7,21 | 144.1 | <0.001 |
| | Time*Predator | 14,42 | 4.3 | <0.001 | 14,42 | 4.9 | <0.001 | 14,42 | 1.3 | 0.265 | 14,42 | 2.8 | 0.005 |
| Responsiveness | Predator | 2,27 | 1.3 | 0.279 | 2,27 | 0.4 | 0.679 | 2,27 | 3.0 | 0.070 | 2,27 | 4.5 | 0.020 |
| | Time | 7,21 | 8.1 | <0.001 | 7,21 | 14.8 | <0.001 | 7,21 | 2.2 | 0.080 | 7,21 | 2.5 | 0.053 |
| | Time*Predator | 14,42 | 0.5 | 0.891 | 14,42 | 1.1 | 0.353 | 14,42 | 1.5 | 0.144 | 14,42 | 1.6 | 0.110 |

[1]Activity was the mean percent of individuals that were moving in the water column or displaying movement of their tail; responsiveness was the mean percent of individuals that responded to the external stimulus of tapping on the holding container.

[2]Predator = no exposure and exposure to either *Anax* or *Belostoma* cues; time = number of days from the start of the experiment.

[3]*F*-test from the repeated-measures analysis-of-variance.

Table A.2. Results of repeated-measures ANOVAs testing the effects of predator cue and ranavirus exposure on the behavioral responses of 4 tadpole species over time.

| Behavior[1] | Main Effect Divisions[2] | Effects[3] | *Hyla chrysoscelis* | | | *Pseudacris feriarum* | | | *Lithobates sylvaticus*[5] | | | *Lithobates clamitans* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | df[4] | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Activity | Between Subjects | Predator | 2,24 | 118.3 | <0.001 | 2,24 | 89.2 | <0.001 | 2,24 | 51.2 | <0.001 | 2,24 | 16.6 | <0.001 |
| | | Virus | 1,24 | 4.9 | 0.037 | 1,24 | 1.7 | 0.204 | 1,24 | 0.1 | 0.767 | 1,24 | 5.7 | 0.026 |
| | | Predator*Virus | 2,24 | 0.1 | 0.886 | 2,24 | 0.6 | 0.555 | 2,24 | 0.2 | 0.810 | 2,24 | 0.3 | 0.740 |
| | Within Subjects | Time | 15,10 | 38.7 | <0.001 | 17,8 | 14.7 | <0.001 | 7,18 | 17.9 | <0.001 | 9,16 | 23.1 | <0.001 |
| | | Time*Predator | 30,20 | 2.1 | 0.048 | 34,16 | 4.1 | 0.002 | 14,36 | 1.8 | 0.070 | 18,32 | 1.3 | 0.277 |
| | | Time*Virus | 15,10 | 2.5 | 0.071 | 17,8 | 0.8 | 0.664 | 7,18 | 2.8 | 0.039 | 9,16 | 1.5 | 0.218 |
| | | Time*Predator*Virus | 30,20 | 1.4 | 0.237 | 34,16 | 1.3 | 0.314 | 14,36 | 2.8 | 0.007 | 18,32 | 1.2 | 0.338 |
| Responsiveness | Between Subjects | Predator | 2,24 | 0.1 | 0.898 | 2,24 | 0.3 | 0.734 | 2,24 | NT | NT | 2,24 | 1.0 | 0.379 |
| | | Virus | 1,24 | 5.7 | 0.025 | 1,24 | 6.9 | 0.015 | 1,24 | 1.0 | 0.325 | 1,24 | 0.4 | 0.516 |
| | | Predator*Virus | 2,24 | 1.2 | 0.323 | 2,24 | 1.5 | 0.244 | 2,24 | 0.7 | 0.521 | 2,24 | 2.7 | 0.087 |
| | Within Subjects | Time | 15,10 | 2.5 | 0.073 | 17,8 | 4.2 | 0.022 | 7,18 | 1.74 | 0.162 | 9,16 | 2.5 | 0.056 |
| | | Time*Predator | 30,20 | 0.6 | 0.878 | 34,16 | 1.5 | 0.189 | 14,36 | 0.3 | 0.996 | 18,32 | 1.6 | 0.118 |
| | | Time*Virus | 15,10 | 0.7 | 0.734 | 17,8 | 0.9 | 0.605 | 7,18 | 2.0 | 0.119 | 9,16 | 1.3 | 0.331 |
| | | Time*Predator*Virus | 30,20 | 0.6 | 0.932 | 34,16 | 1.9 | 0.095 | 14,36 | 0.7 | 0.774 | 18,32 | 0.7 | 0.827 |

Table A.2 (continued).

---

[1]Activity was the mean percent of individuals that were moving in the water column or displaying movement of their tail; responsiveness was the mean percent of individuals that responded to the external stimulus of tapping on the holding container.

[2]Between- and within-subject tests for the repeated-measures analysis-of-variance (ANOVA).

[3]Predator = no exposure and exposure to either *Anax* or *Belostoma* cues; virus = no exposure and exposure to ranavirus; time = number of days from the start of virus exposure.

[4]Degrees of freedom differ among species for some effects because experimental units were removed from the analysis due to mortality.

[5]NT = no test performed because all individuals responded the same to all three predator treatments, hence no variation existed for ANOVA.

Table A.3. Results of analyses testing the effects of predator cue and ranavirus exposure on the survival, growth, and viral load of tadpoles.

| Response Variable[1] | Effects[2] | *Hyla chrysoscelis* | | | *Pseudacris feriarum* | | | *Lithobates sylvaticus*[3, 4] | | | *Lithobates clamitans* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | df | $\chi^2$ | *P* | df | $\chi^2$ | *P* | df | $\chi^2$ | *P* | df | $\chi^2$ | *P* |
| Survival | Predator | 2 | 0.5 | 0.763 | 2 | 0.3 | 0.861 | | NT | | 2 | 0.6 | 0.745 |
| | Virus | 1 | 17.9 | <0.001 | 1 | 12.6 | <0.001 | | NT | | 1 | 24.4 | <0.001 |
| | | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* | df | *F* | *P* |
| Growth | Predator | 2,24 | 2.6 | 0.092 | 2,24 | 0.6 | 0.577 | 2,14 | 1.396 | 0.285 | 2,24 | 2.0 | 0.152 |
| | Virus | 1,24 | 1.2 | 0.278 | 1,24 | 1.7 | 0.207 | | NT | | 1,24 | 12.1 | 0.002 |
| | Predator*virus | 2,24 | 1.1 | 0.356 | 2,24 | 2.3 | 0.127 | | NT | | 2,24 | 2.3 | 0.119 |
| Viral load | Predator | 2,46 | 1.3 | 0.278 | 2,46 | 1.4 | 0.266 | | NT | | 2,52 | 0.7 | 0.522 |

[1]Survival was the mean number of individuals per treatment that survived to the end of the experiment; growth was the mean growth of each experimental unit; viral load was back calculated from the mean CT value of the qPCR then log transformed.

[2]Predator*virus effect was not included for survival because a Kruskal-Wallis test was used due to non-normal data; interaction of main effects cannot be tested using this non-parametric test. Virus effect was not included for viral load, because all unexposed individual were negative for ranavirus.

[3]A one-way analysis-of-variance was conducted on growth because all virus-exposed individuals died.

[4]NT = no test performed, because all individuals in the virus-exposed treatment died.

Table A.4. Egg mass collection sites for all experiments.

| Scientific Name | State | County | Location | Lat - Long | UTM | # Egg masses |
|---|---|---|---|---|---|---|
| *Anaxyrus americanus* | PA | Crawford | Pymatuning State Park | 41°34'10"N, 80°27'20"W | 17 545392E 4602117N | 10 |
| *Hyla chrysoscelis* | TN | Knox | Private landowner | 36°01'30"N, 83°47'30"W | 17 248426E 3990338N | 9 |
| *Lithobates clamitans* | TN | Union | Chuck Swan WMA | 36°21'29"N, 83°54'49"W | 17 238539E 4027616N | 7 |
| *Lithobates pipiens* | PA | Crawford | Pymatuning State Park | 41°41'30"N, 80°30'20"W | 17 541146E 4615661N | 10 |
| *Lithobates sylvaticus* | TN | Knox | Royal Blue WMA | 36°02’10"N, 83°51’19"W | 17 242745E 3991727N | 9 |
| *Pseudacris feriarum* | TN | Knox | Seven Islands Wildlife Refuge | 35°56'59"N, 83°41'41"W | 17 256940E 3981756N | 20 |
| *Scaphiopus holbrookii* | TN | Union | Chuck Swan WMA | 36°21'29"N, 83°54'49"W | 17 238539E 4027616N | 20 |

## Figure Legends

Figure A.1. The effects of predator cues on the activity (A) and responsiveness (B) of *Hyla chrysoscelis* (HYCH), *Pseudacris feriarum* (PSFE), *Lithobates sylvaticus* (LISY), and *L. clamitans* (LICL) tadpoles prior to the addition of virus. Predator cue treatments are no-predator (NP), *Belostoma* (B), and *Anax* (A). Data (least-squares means $\pm$ 1 SE) were averaged across time.

Figure A.2. The effects of predator cues and virus addition on the activity (A) and responsiveness (B) of *Hyla chrysoscelis* (HYCH), *Pseudacris feriarum* (PSFE), *Lithobates sylvaticus* (LISY), and *L. clamitans* (LICL) tadpoles. Predator cue treatments are no predator (NP), *Belostoma* (B), and *Anax* (A). Open circles represent the no-virus treatment and closed circles represent the virus treatment. Data (least-squares means $\pm$ 1 SE) were averaged across time.

Figure A.3. The effects of predator cues and virus addition on the survival (A) and growth (B) of *Hyla chrysoscelis* (HYCH), *Pseudacris feriarum* (PSFE), *Lithobates sylvaticus* (LISY), and *L. clamitans* (LICL) tadpoles. Predator cue treatments are no predator (NP), *Belostoma* (B), and *Anax* (A). Open circles represent the no-virus treatment and closed circles represent the virus treatment. Data (least-squares means $\pm$ 1 SE) were averaged across time. Given that all *L. sylvaticus* tadpoles exposed to the virus died, I only tested for differences in growth among predators for the no-virus treatment.

Figure A.4. Percent mortality and infection averaged across species for each developmental stage (A) and averaged across developmental stage for each species (B). Developmental stages are as follows embryo, hatchling (Hatch), larval, and metamorphosis (Meta). Species codes are as follows *Pseudacris feriarum* (PSFE), *Anaxyrus americanus* (ANAM), *Hyla chrysoscelis* (HYCH), *Lithobates pipiens* (LIPI), *L. clamitans* (LICL), *Scaphiopus holbrookii* (SCHO), and *L. sylvaticus* (LISY). Similar shaded bars with unlike letters are different ($P < 0.001$) by logistic analysis; $n = 80$ and 140 per species and developmental stage, respectively.

Figure A.5. Relationship of mortality and infection rates for 22 experimental trials testing the susceptibility of 7 North American anuran species to ranavirus among 4 developmental stages.

Figure A.6. Mean viral load (log μg/ml) of individuals infected with ranavirus for *Pseudacris feriarum* (PSFE, $n = 15$), *Anaxyrus americanus* (ANAM, $n = 10$), *Hyla chrysoscelis* (HYCH, $n = 22$), *Lithobates pipiens* (LIPI, $n = 35$), *L. clamitans* (LICL, $n = 38$), *Scaphiopus holbrookii* (SCHO, $n = 52$), and *L. sylvaticus* (LISY, $n = 76$). Data are averaged across developmental stages. Bars with unlike letters are different ($P < 0.05$) by Tukey's HSD test.

Figure A.7. Percent mortality and infection among embryo, hatchling, larval, and metamorphosis developmental stages for *Lithobates sylvaticus* (LISY, A), *L. pipiens* (LIPI, B), *L. clamitans* (LICL, C), *Anaxyrus americanus* (ANAM, D), *Pseudacris feriarum* (PSFE, E), *Hyla chrysoscelis* (HYCH, F), and *Scaphiopus holbrookii* (SCHO, G). Similar shaded bars with unlike letters are different ($P < 0.006$) by chi-square test of homogeneity; $n = 20$ per developmental stage for each species.

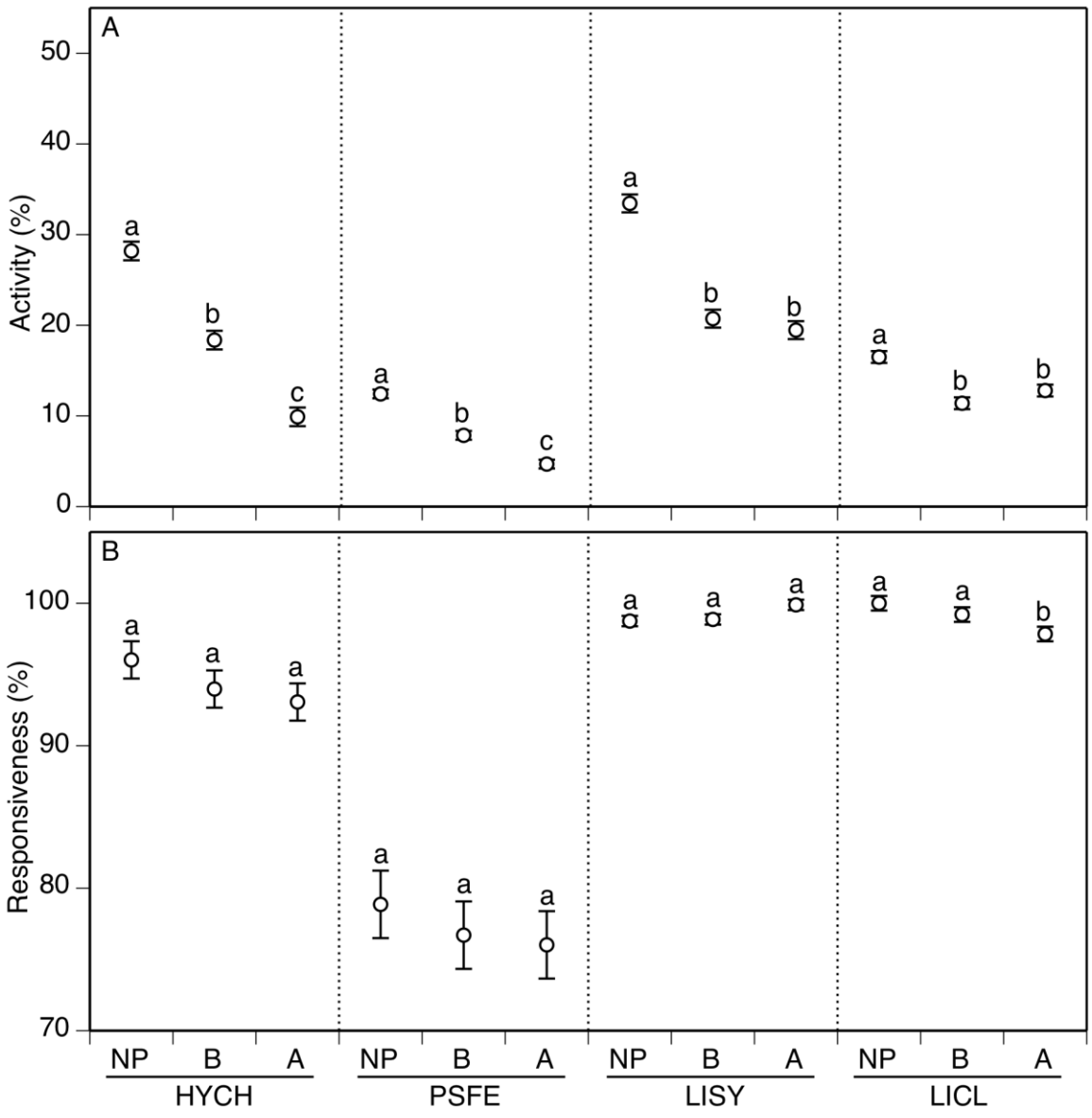


**Figure A.1**

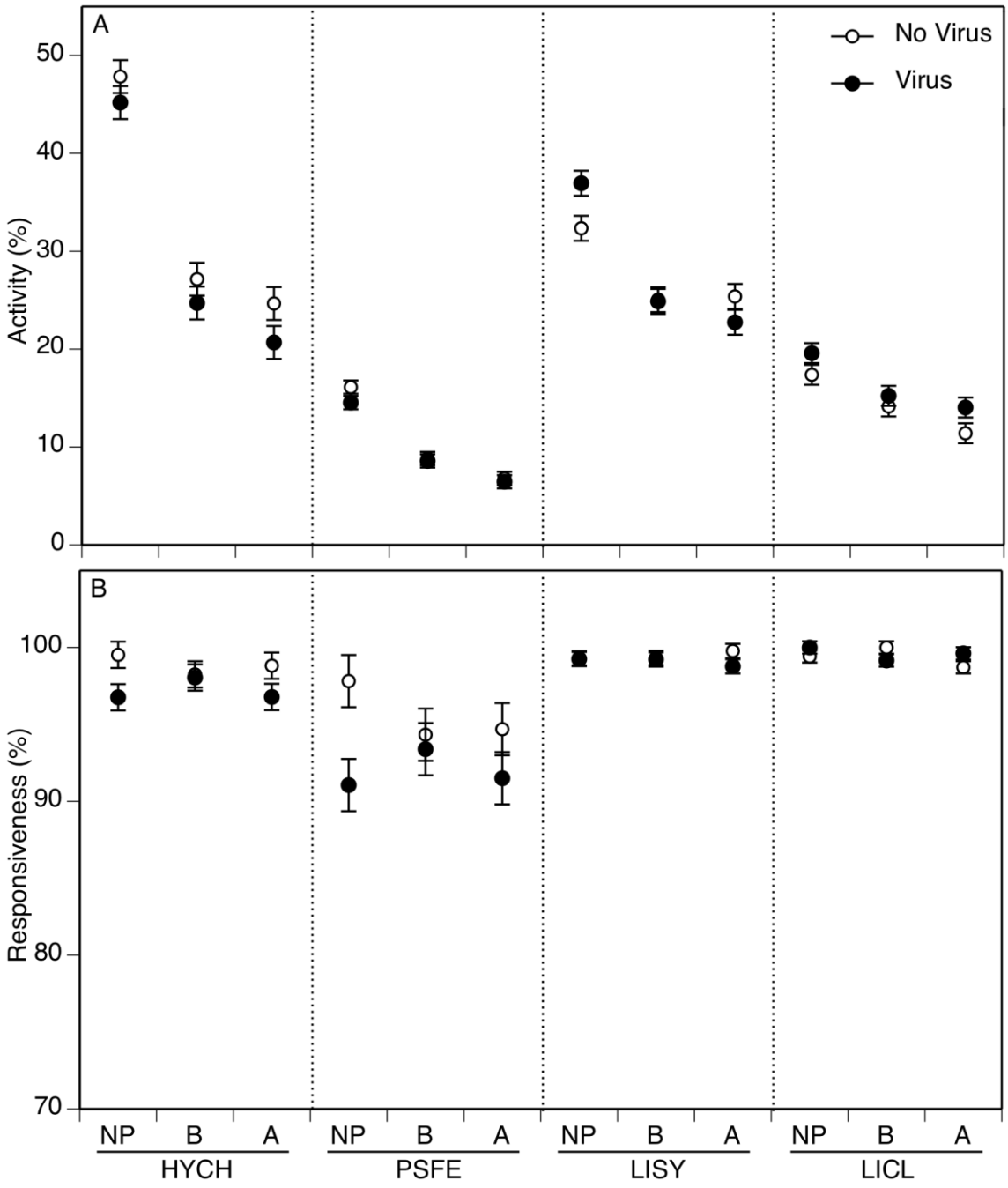
A
No Virus
Virus
Activity (%)
50
40
30
20
10
0
B
Responsiveness (%)
100
90
80
70
NP
B
A
HYCH
PSFE
LISY
LICL

**Figure A.2**

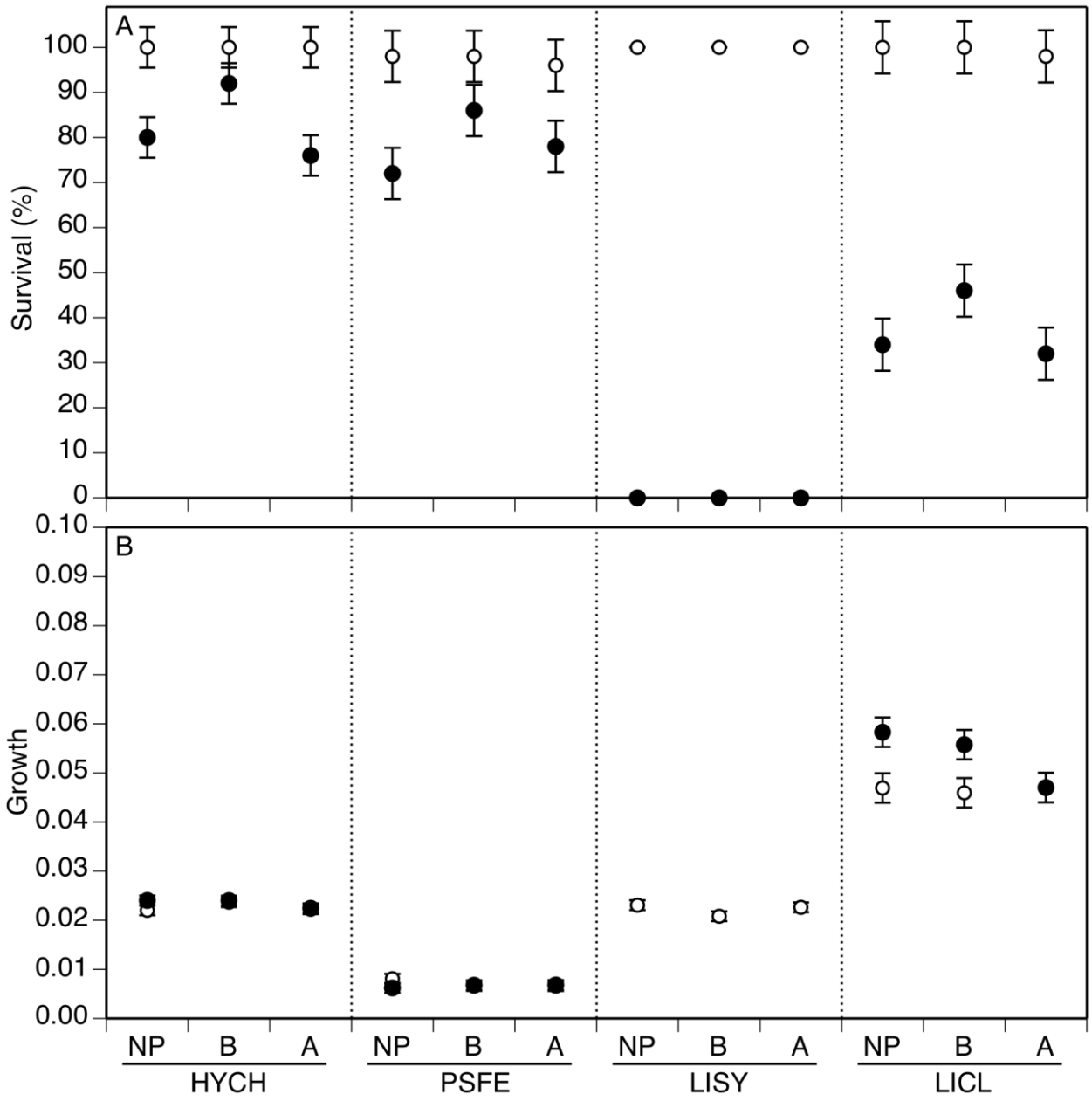
A
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
B
Growth
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
NP
B
A
HYCH
PSFE
LISY
LICL

**Figure A.3**

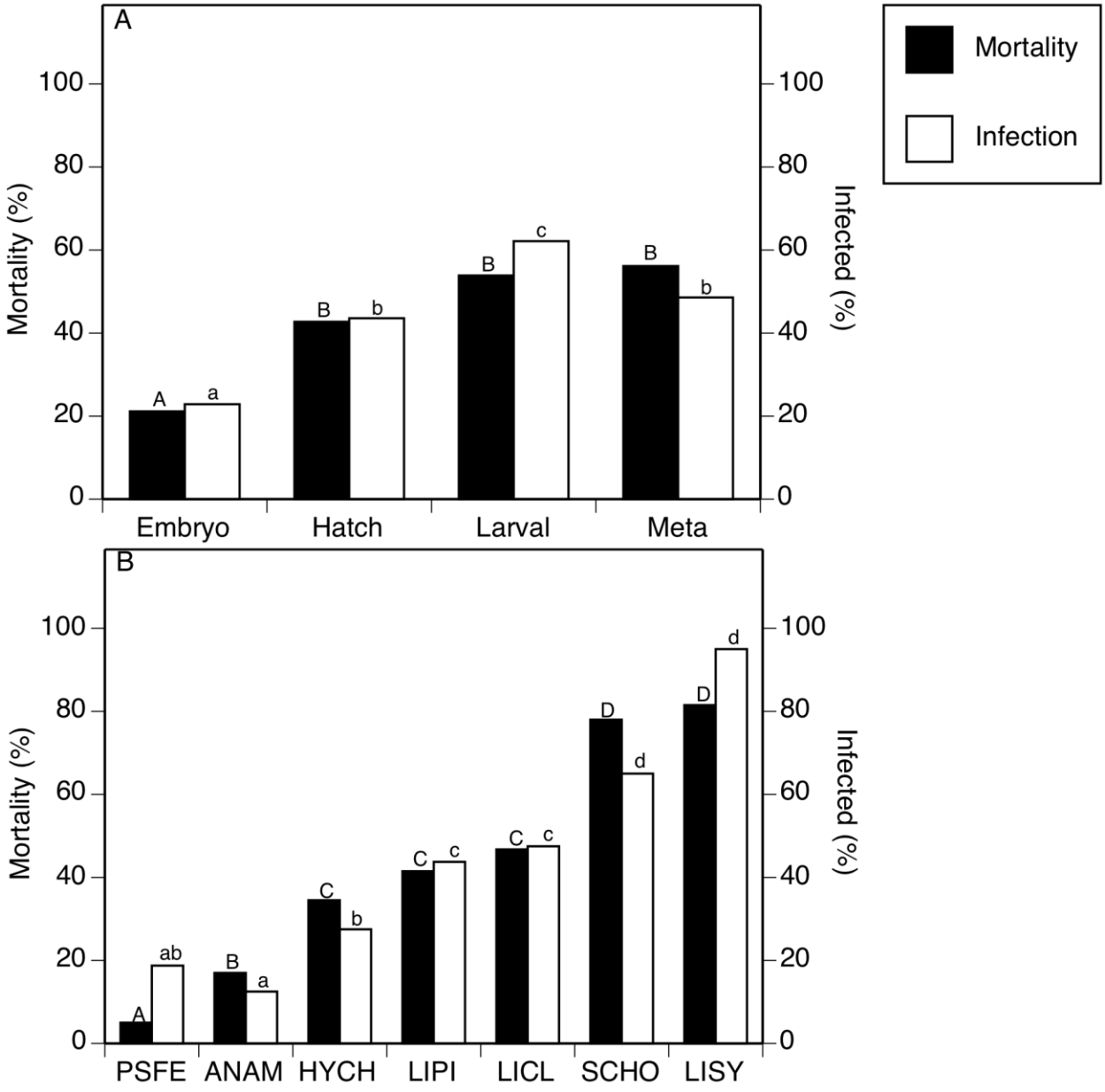
A
Mortality
Infection
Mortality (%)
Infected (%)
100
80
60
40
20
0
A
a
B
b
B
c
B
b
Embryo
Hatch
Larval
Meta
B
A
ab
B
a
C
b
C
c
C
c
D
d
D
d
PSFE
ANAM
HYCH
LIPI
LICL
SCHO
LISY

**Figure A.4**

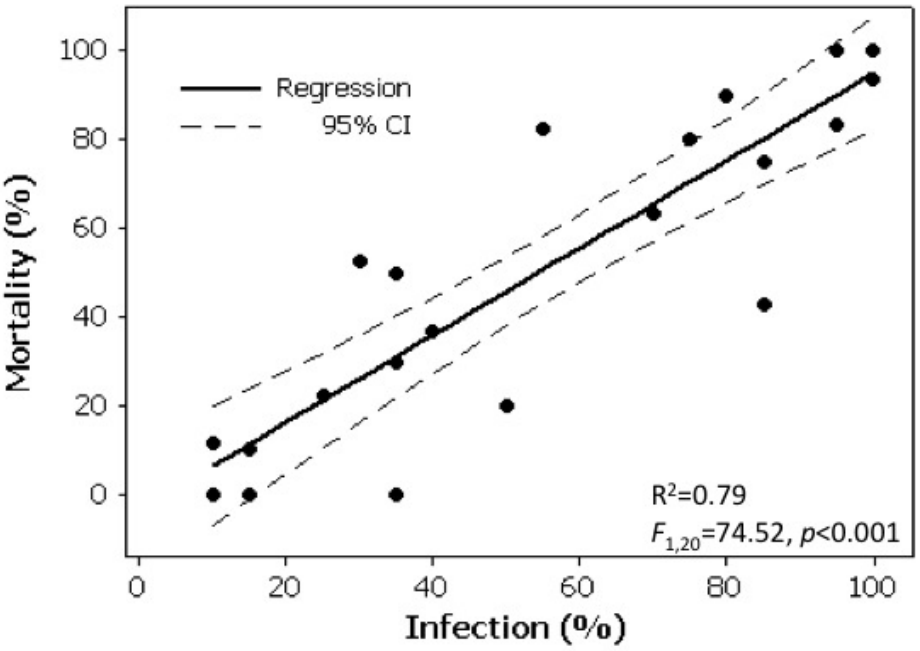


**Figure A.5**

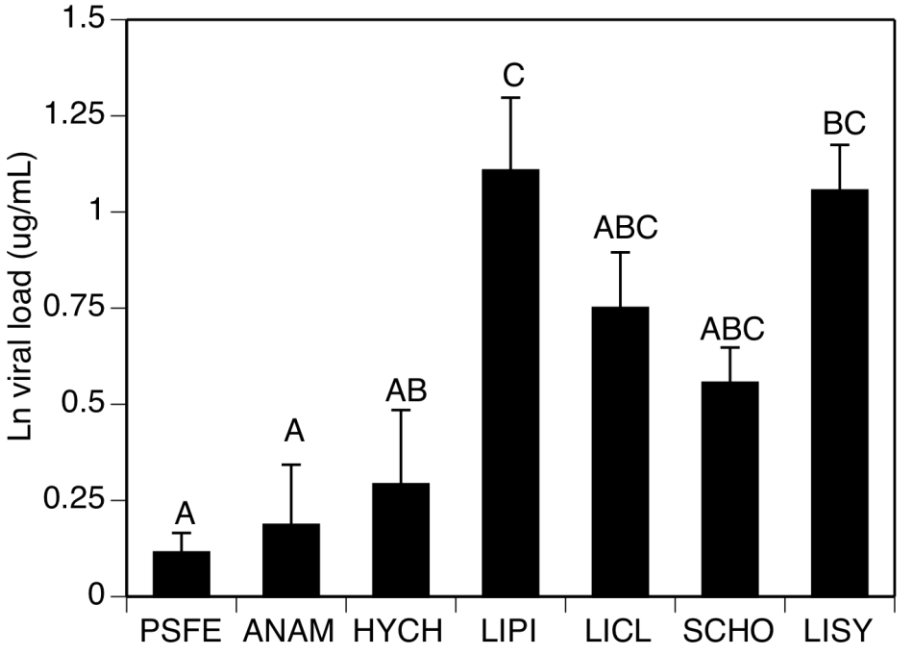


**Figure A.6**

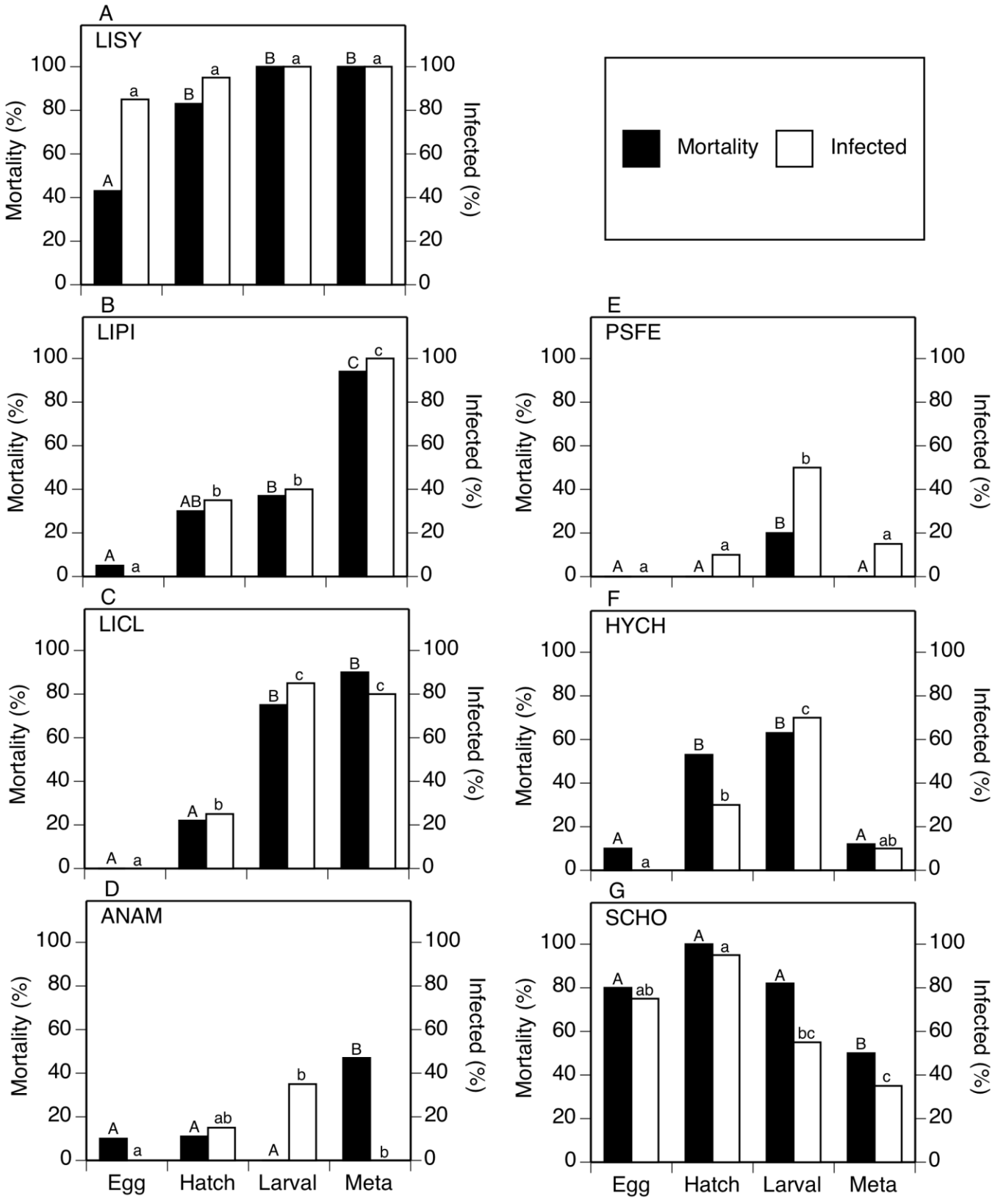
A
LISY
B
LIPI
C
LICL
D
ANAM
E
PSFE
F
HYCH
G
SCHO
Mortality
Infected
Mortality (%)
Infected (%)
Egg
Hatch
Larval
Meta

**Figure A.7**

# APPENDIX B

# INTERACTIONS OF TIME WITH PREDATOR AND VIRUS TREATMENTS

Prior to ranavirus exposure, predator and time effects interacted for percent activity of *Hyla chrysoscelis*, *Pseudacirs feriarum*, and *Lithobates clamitans* (Table A.1). The interaction for *P. feriarum* was driven by the variability in the *Belostoma* treatments as tadpole activity was similar to the *Anax* treatment during days 1, 2, 3, 7, and 8 ($P \geq 0.057$), but significantly greater in the *Belostoma* treatment for days 4 – 6 ($P \leq 0.02$, Figure B.1a). Similarly, the interaction for *H. chrysoscelis* was driven by changes in the *Belostoma* treatment means as tadpole activity was similar to *Anax* treatments during days 2, 3, and 6 ($P \geq 0.08$), but were greater than *Anax* treatments for all other days ( $P \leq 0.04$, Figure B.1b). The interaction for *L. clamitans* was driven by no differences between the *Belostoma* and *Anax* at the beginning and end of the experiment ($P \geq 0.25$), but during day 4 activity was significantly greater in the *Belostoma* treatment compared to the *Anax* treatment ($P \leq 0.001$, Figure B.1c).

After exposure to ranavirus, predator and time effects interacted for percent activity of *Hyla chrysoscelis* and *Pseudacris feriarum* (Table A.2). Similar to the pre-exposure results, the interaction for *P. feriarum* was driven by variability in the activity levels of *Belostoma* treatments. During days 5 and 10, tadpole activity in the *Belostoma* treatments was significantly greater than in the *Anax* treatments ($P \leq 0.02$), but activity was similar between the predator treatments for the remaining days of the experiment ($P \geq 0.07$, Figure B.2a). For *H. chrysoscelis,* the interaction was driven by significantly greater activity in the *Belostoma* treatments on days 2, 3, and 11 compared to the *Anax* treatments ($P \leq 0.02$); no differences were detected between predator treatments for the remaining days ($P \geq 0.11$, Figure B.2b).

There was a 3-way interaction with time, virus, and predator effects and a 2-way interaction between time and virus effects for percent activity of *Lithobates sylvaticus* (Table A.2), thus I separated the analyses by virus and predator treatments to identify the trends (Figure

B.3). Generally, between days 3 and 7, within the no virus and virus treatments, both predator species were reducing activity levels when compared to the no predator treatments ($P \leq 0.10$, Figure B.3).

## Figure Legends

Figure B.1. Percent activity for *Pseudacris feriarum* (A), *Hyla chrysoscelis* (B), and *Lithobates clamitans* (C) during 8 consecutive days prior to ranavirus exposure. Predator cues treatments were *Anax* (square), *Belostoma* (circle), and no predator (triangle). There was a significant interaction ($P < 0.005$) between predator treatment and time. Data are least-squares means $\pm$ 1 SE

Figure B.2. Percent activity for *Pseudacris feriarum* (A) and *Hyla chrysoscelis* (B) during 18 and 16 days post-exposure to ranavirus, respectively. Predator cue treatments were *Anax* (square), *Belostoma* (circle), and no predator (triangle). There was a significant interaction ($P < 0.05$) between the predator treatment and time. Data (least-squares means $\pm$ 1 SE) were averaged across virus treatments

Figure B.3. Percent activity for *Lithobates sylvaticus* during 8 days post-exposure to ranavirus. Given the significant predator*virus*time interaction, percent activity is display for the predator treatments within each virus treatment over time. Predator cue treatments were *Anax* (square), *Belostoma* (circle), and no predator (triangle). Data are least-squares means $\pm$ 1 SE.

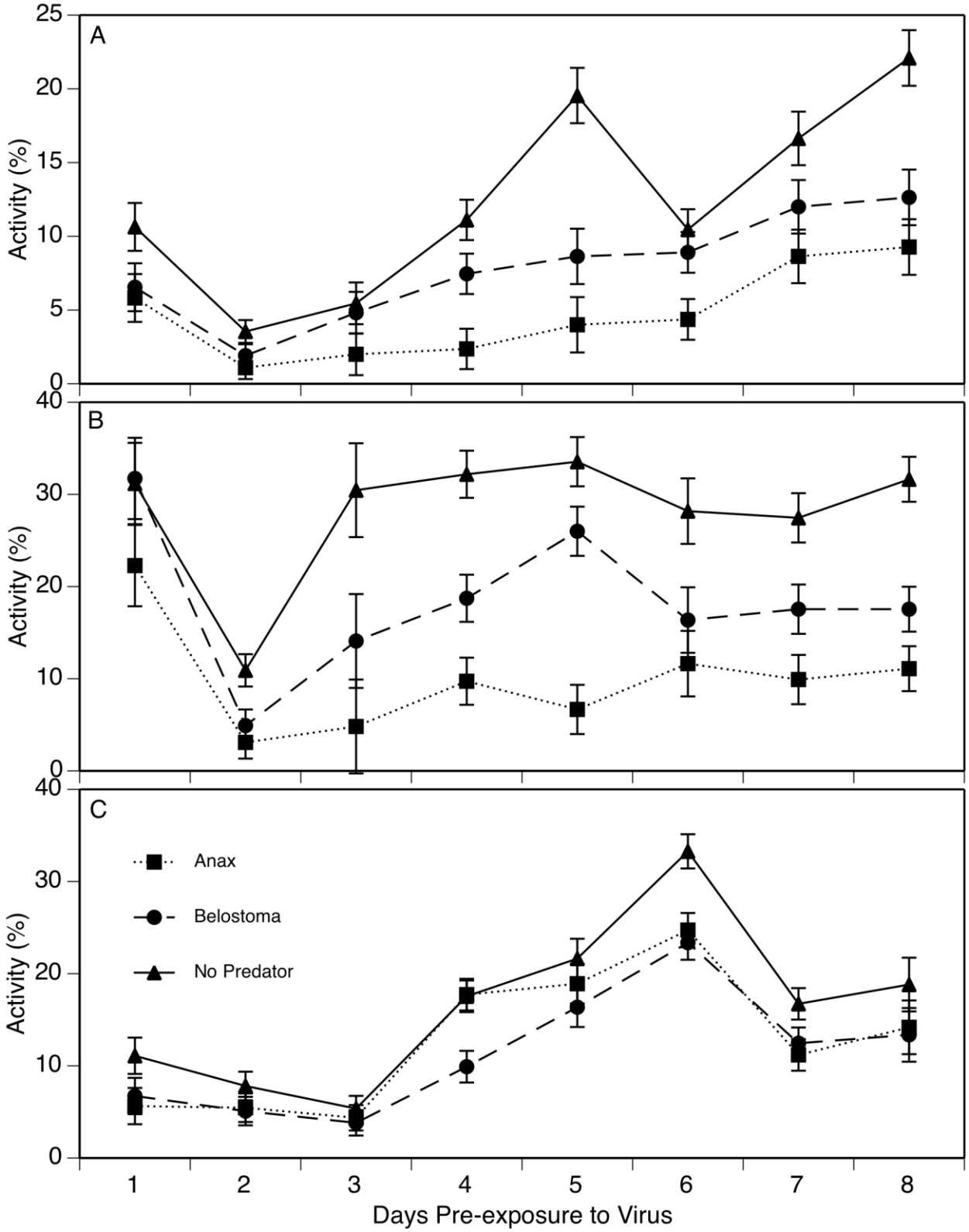
A
B
C
Activity (%)
Anax
Belostoma
No Predator
Days Pre-exposure to Virus

**Figure B.1**

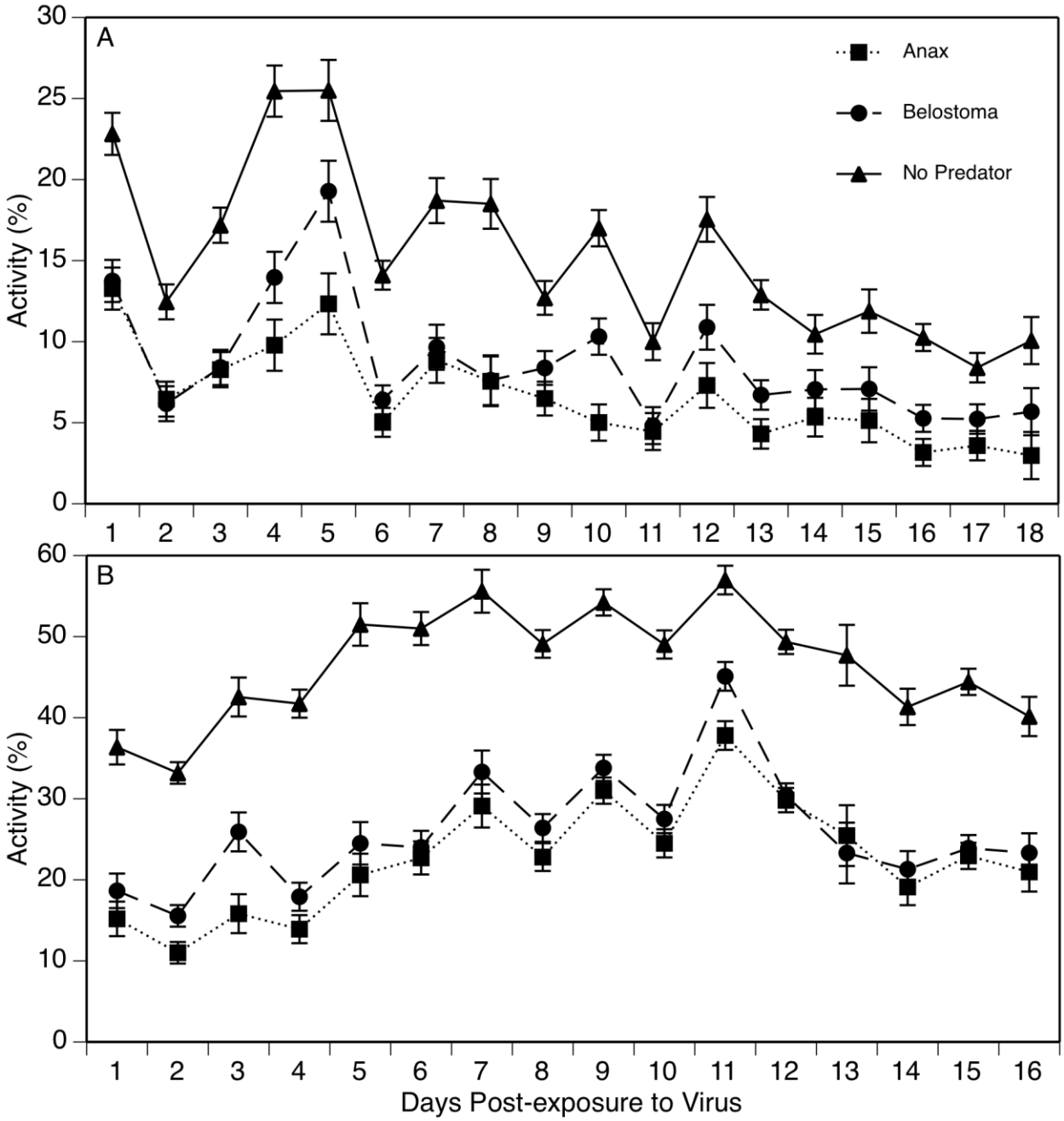
A
Anax
Belostoma
No Predator
Activity (%)
B
Activity (%)
Days Post-exposure to Virus

**Figure B.2**

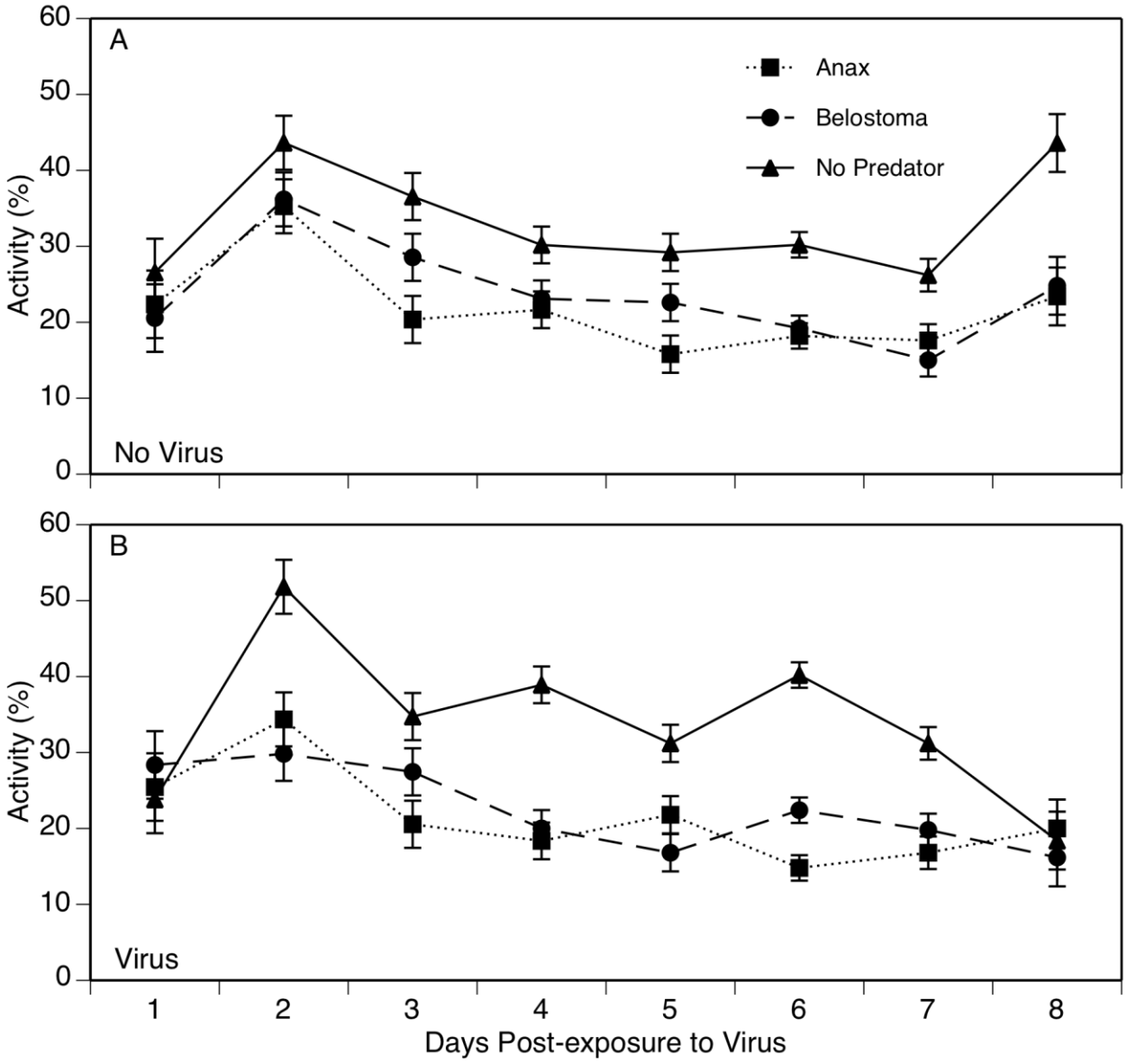
A
Anax
Belostoma
No Predator
Activity (%)
No Virus
B
Activity (%)
Virus
Days Post-exposure to Virus

**Figure B.3**

## Vita

Nathan Haislip was born in Lewisburg, TN to the parents of Michael and Cindy Haislip. He attended Marshall County High School where he graduated with high honors. Nathan obtained his Bachelor's degree in 2008 with Magna cum laude honors majoring in Wildlife and Fisheries Management with a minor in forestry at the University of Tennessee-Knoxville. Throughout his undergraduate career he was active in both field and laboratory research conducting several independent studies and internships on various herpetofauna. In June of 2008, he accepted a graduate student teaching assistantship at the University of Tennessee-Knoxville where he received a Master's in Wildlife and Fisheries Science with a minor in statistics in 2010. He is currently working at the Fort Worth Zoo in the herpetology department as an animal keeper.