EFFECTS OF MULTIPLE STRESSORS ON AMPHIBIAN PATHOGEN PREVALENCE AND SUSCEPTIBILITY

by

MEGAN ELIZABETH WINZELER

(Under the Direction of Stacey Lance)

ABSTRACT

Amphibians experience multiple abiotic and biotic stressors throughout development. How these stressors interact with each other is complex and hard to understand, particularly when occurring in combination. To determine the interactions of natural hydroperiod length and amphibian pathogens, I studied ranavirus and *Batrachochytrium dendrobatidis* (Bd) in 20 wetlands on the Savannah River Site (SRS) in two Ambystomatid salamanders. I showed that during a 2 year study, presence and prevalence of both pathogens vary by year. I used a 2x2x2 factorial design to study the interactions of copper, shortened hydroperiod, and exposure to ranavirus. I found that sublethal impacts on growth due to exposure to a pathogen can occur, even without recorded effects of other stressors. Our study highlights the need to include multi-year surveillance of amphibian populations, due to the potential annual pathogen dynamic cycles and sublethal effects of exposure to the pathogen in amphibians.

INDEX WORDS: Amphibian; *Batrachochytrium dendrobatidis* (Bd); Copper; Disease; Hydroperiod; Multiple stressors; Ranavirus

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Wildlife stressors are any abiotic or biotic factor that causes a physiological change in an individual (Johnstone et al. 2012) and can be natural or anthropogenic in nature. Predation, wetland drying, and competition are examples of common natural stressors (Reeves et al. 2011; Amburgey et al. 2012; Atobe et al. 2014). Heavy-metal contamination, pesticides, and habitat destruction are anthropogenic stressors that can cause stress on wildlife and their populations (Hopkins et al. 1997; Cushman 2006). Typically, these stressors occur together, causing additive, synergistic, and antagonistic effects on wildlife populations. Wildlife response to the combination of stressors can include physical, such as morphological (Schoeppner and Relyea 2008; Márquez-García et al. 2009), and physiologic, like growth and development (Browne et al. 2003; Gomez-Mestre et al. 2013), changes. In some cases, response to stressors can alter immune function (Carey et al. 1999; Hawley and Altizer 2011) as well as the epidemiology of some diseases (Jones et al. 2008).

AMPHIBIAN STRESSORS AND DECLINES

Amphibians are under global investigation due to recent population declines caused by many factors, including habitat loss, disease, contaminants, climate change and invasive species (Daszak et al. 2003a). These factors often work in combination to impact an amphibian population but few studies combine more than two stressors, leading to a

large void in our ability to understand and appropriately manage amphibian populations. Natural history characteristics, like a bi-phase life cycle (Wilbur and Collins 1973), make amphibians vulnerable to alterations in the surrounding ecosystem (Wake and Vredenburg 2008). An individual can experience a stressor early in life as larvae in the aquatic environment that impacts development and health at the terrestrial life stage (Berven 1990; Van Buskirk and Saxer 2001). Amphibian larvae experiencing stressors like pond-drying or increased larval density could have increased time to metamorphosis (Boone et al. 2007), and decreased survival and growth (Skelly 1996). Predator presence can slow larval growth and activity, potentially causing sublethal impacts on development (Lima and Dill 1990; Bridges 1999). Broadening the focus of these studies to include multiple stressors, like wetland length, presence of contaminants, and disease risk, at both the larval and terrestrial life stages is critical to understanding the full scope of amphibian population declines.

AMPHIBIAN COMPLEX LIFE CYCLES

Part of amphibians' complex life cycle includes a developmental period as aquatic larvae, then metamorphosis into terrestrial adults (Wilbur and Collins 1973). During aquatic development, plasticity allows for individuals within species-specific size constraints to respond to environmental conditions that can vary from year to year, like food resources and wetland hydroperiod (Wilbur 1980; Morey and Reznick 2000). A plastic response to metamorphose early and migrate to land where more resources are located can be beneficial when a wetland begins to dry or there are high densities of conspecifics (Wilbur 1980). This ability comes at a physiologic cost, with individuals

emerging at smaller sizes (Newman 1988; Searcy et al. 2014) or with weakened immune response (Gervasi and Foufopoulos 2007; Haislip et al. 2011; Searle et al. 2013). Metamorphosis is controlled by hormones in the thyroid and interrenal axes through the stress hormone corticosterone (CORT; Denver 1997; Denver 1998). Total water volume in a wetland, temperature, density of conspecifics, and resource availability are all hypothesized as indicators for amphibian metamorphosis (Berven 1982; Wilbur 1987; Tejedo and Reques 1994; Denver et al. 1998). Changes to these factors can cause alterations in amphibian complex life cycles and latent effects in future life stages (Pechenik 2006).

HYDROPERIOD

The amount of time a wetland holds water, its hydroperiod (Ryan and Winne 2001), is critical to successful larval amphibian development because it can affect time to metamorphosis and overall survival (Richter-Boix et al. 2011). The effect of hydroperiod on an individual's time to metamorphosis is well documented (Newman 1988; Semlitsch and Wilbur 1988; Newman 1989). At three wetlands on the Savannah River Site (SRS), the longer a wetland stayed wet the greater amount of amphibians were able to successfully metamorphose (Pechmann et al. 1989). Hydroperiod can also affect an individual's immune system response when experiencing desiccation. Wood frogs had lower total leucocyte counts, a weaker immune response to PHA, and shorter developmental times (Gervasi and Foufopoulos 2007) when hydroperiod was shortened. Larval amphibians exposed to accelerated pond-drying conditions are more likely to

emerge early (Koprivnikar et al. 2014), but some amphibian species are adapted to short hydroperiod lengths (Denver 2009).

Short hydroperiod wetlands are at a greater risk of early drying, increasing desiccation risk (Pechmann et al. 1989), than wetlands with longer hydroperiods. In some years, a shortened hydroperiod can result in catastrophic reproductive failure when the wetland dries before any larvae successfully metamorphose (Taylor et al. 2006). Tadpoles experiencing early pond drying had lower mass and shorter limbs than individuals of the same species not exposed to pond drying (Gomez-Mestre et al. 2013). Larval mole salamanders (*Ambystoma talpoideum*), in wetlands that dried later had extended time to metamorphosis leading to a greater number of individuals metamorphosing than in wetlands that dried early (Semlitsch and Wilbur 1988). While responding physiologically to current hydrologic conditions can be beneficial in many cases (i.e. escape predation or poor water quality conditions; Hamer and Parris 2013), the limited energy resources acquired during early life stages can affect later life stages (Crespi and Warne 2013). Accelerated development typically results in reduced size at metamorphosis (Richter-Boix et al. 2011; Johansson and Richter-Boix 2013) which can lead to reduced fitness (Willson et al. 2012). The potential interactive effects of reduced or increased hydroperiod and amphibian pathogen dynamics could play a significant role in amphibian population fluctuations.

AMPHIBIAN PATHOGENS

One class of stressors that are increasingly studied due to their implications in population and community regulation are amphibian pathogens (Daszak et al. 1999).

Pathogens can affect an individual in many ways, from sublethal malformations to mortality (Blaustein et al. 2011). It is hypothesized that the sudden emergence of amphibian pathogens is partly due to a weakened host immune response resulting from exposure to environmental contaminants (Christin et al. 2003; Rohr et al. 2008). Environmental changes can influence how the pathogen affects the amphibian host, as well as the prevalence in the population. These environmental changes can be either biotic or abiotic stressors that can cause synergistic, additive, or antagonistic effects when occurring together. The understanding of amphibian pathogens is a critical conservation issue due to the declines in worldwide populations (Daszak et al. 1999) and long-term studies can aid in this effort.

RANAVIRUS

Ranaviruses (Family: *Iridoviridae*) are a group of viruses that infect ectothermic vertebrates across 6 continents and 175 species (Duffus et al. 2015). Morbidity and mortality of amphibians have been attributed to ranaviruses, but no direct species extinctions have been reported. Ranaviruses cause symptoms such as lethargy, emaciation, hemorrhaging, and death to amphibians that breed in lentic systems, like lakes and ephemeral wetlands (Harp and Petranka 2006). Two species of ranaviruses, frog virus 3 (FV3) and *Ambystoma tigrinum* virus (ATV) are the focus of studies in the United States and primarily infect amphibians (reviewed in Miller et al. 2011). Outbreaks of ranaviruses in amphibian populations typically occur late in the larval stage and newly metamorphosed individuals (Green et al. 2002). In Britain, ranavirus prevalence increases with population density, fish presence, and increased pond depth along with other abiotic

stressors that are anthropogenic (North et al. 2015). Ranavirus does have a seasonal pattern in most locations, with die-off events usually occurring in late spring or summer (Grizzle and Brunner 2003; Hoverman et al. 2012), when most amphibians begin final stages of metamorphosis as temperatures increase. Sublethal infections of ranaviruses can cause negative impacts on growth and development (Echaubard et al. 2010) and allow the virus to persist in the environment through adults returning to breed (Brunner et al. 2004).

The interaction of ranavirus exposure with other environmental stressors are not well understood. Exposure to the common pesticide atrazine increased susceptibility of tiger salamanders (*Ambystoma tigrinum*) to ATV and lowered leukocyte levels, suggesting a weakened immune system due to the atrazine exposure (Forson and Storfer 2006). Temperature can also influence ranavirus prevalence in tiger salamanders causing increased mortality and time to death in individuals exposed to 10 and 18°C (Rojas et al. 2005). Prevalence of ranavirus increases as proximity to industry and human housing also increases but agricultural activity had no effect (St-Amour et al. 2008). Additionally, infection with at least two amphibian pathogens can be common (>68%; Hoverman et al. 2012) or uncommon (0-20%; St-Amour et al. 2008; Warne et al. 2016). Understanding how multiple stressors and more broadly outbreaks, affect pathogen susceptibility in amphibian populations is important for future conservation research.

BATRACHOCHITRIUM DENDROBATIDIS **(Bd)**

Batrachochytrium dendrobatidis (Bd) is a fungal pathogen that causes the disease chytridiomycosis in the epidermis of amphibians (Berger et al. 1998; Lips 1999) and occurs in amphibians on every continent where amphibians exist (Lannoo et al. 2011). Bd

will persist in populations through extreme environmental factors like high temperature and increased moisture levels (Piotrowski et al. 2004; Woodhams et al. 2008), but also survives independently of a host through zoospores within an environment (Ron 2005). There is interspecific variation in susceptibility to Bd, both in the larval and adult life stages (Blaustein et al. 2005; Brannelly et al. 2012; Gervasi et al. 2014). Temperature changes can affect Bd reproduction and cause seasonal variation in infection prevalence on the landscape (Berger et al. 1998; Savage and Zamudio 2011). Exposure to Bd can also increase larval period in western toads (*Anaxyrus boreas*), cascades frogs (*Rana cascadae*), American bullfrogs (*Lithobates catesbeianus*; Searle et al. 2014), and wood frogs (*Lithobates sylvatica*; Groner and Relyea 2015). How Bd influences amphibian populations in the presence of other biotic and abiotic stressors is important to understanding the pathogen's complex dynamics.

The effects of multiple stressors on Bd susceptibility is better studied than the effects on ranavirus susceptibility. The combination of predator cues and Bd caused wood frog tadpoles to have reduced infection loads than individuals not exposed to the predator cues (Groner and Relyea 2015). Pacific treefrogs exposed to flutuacting temperature, Bd, and carbaryl, a commonly used pesticide, had decreased prevalence of Bd in the pesticide-only treatments and decreased survival in temperature-only treatments (Rumschlag et al. 2014). Due to varying results of exposure to multiple stressors and Bd, it is important that research focuses on the complex interaction of pathogen, host, and environment.

COPPER

As exposure to contaminants in natural environments increases, the importance of understanding their effects on individual and populations grows (Chen et al. 2004; Ficken and Byrne 2013). Pesticides are commonly studied due to the well-documented effects on amphibians (Bridges and Semlitsch 2000; Hayes et al. 2002; Kiesecker 2002; Boone et al. 2005; Rohr et al. 2008; Budischak et al. 2008; Johnson et al. 2013) and their ubiquitous distribution across the landscape (Boethling et al. 2009). However, heavy metal contamination is another common contaminant that is less well studied then pesticides. As heavy metal distribution increases on the landscape through surface waters (Wang et al. 2014) and constructed mitigation wetlands (Lance et al. 2012; Lance et al. 2013), it is increasingly important to understand the effects on amphibian populations. Chronic exposure to heavy metals can occur through repeated applications or continual discharge into wetlands (Simpson et al. 2013). Heavy metals affect amphibians during larval development and growth (Carey and Bryant 1995). Aluminum in combination with low pH caused reduced growth and slower development in gray treefrog larvae (Jung and Jagoe 1995). Heavy metals common in coal mine by-products, like arsenic and antimony, can also bioaccumulate within the food chain through amphibian tadpoles (Dovick et al. 2016).

Due to its pervasive distribution and toxicity at sub-lethal concentrations, copper is ranked the second highest contaminant risk to amphibians (Fedorenkova et al. 2012). Exposure to Cu increased larval period in southern leopard frogs (*Lithobates sphenocephalus*; Lance et al. 2012) and delayed development in the eastern narrowmouth toad (*Gastrophyrne carolinensis*; Flynn et al. 2015). Sub-lethal effects of copper include

reduced activity including foraging and predator avoidance (Hayden et al. 2015), malformations (Aronzon et al. 2011), increased time to tail resorption and limb development (Peles 2013), and increase larval period (García-Muñoz et al. 2009). These effects can present themselves on the population level, causing changes in population dynamics (Willson et al. 2012) and potentially long-term population viability.

THESIS OBJECTIVES

It is important to begin understanding the influence of common amphibian stressors, like hydroperiod length, heavy metal contamination, and pathogen exposure, on multiple levels. To do this, I focused on determining how multiple stressors influence prevalence of two amphibian pathogens, Bd and ranavirus, and how the interaction of multiple stressors impact larval susceptibility. To address my first question, in Chapter 1, I developed a large-scale field study to study population-level pathogen prevalence in two salamander species in wetlands of differing hydroperiod length. My objectives were to 1) examine how wetland hydroperiod affects prevalence of Bd and ranaviruses and 2) to determine if prevalence differed between two species of salamander. In Chapter 2, I use a controlled experiment to understand how early exposure to two stressors, shortened hydroperiod and copper, affects susceptibility to ranavirus. My objectives were to examine the effects of early exposure to shortened hydroperiod and copper on 1) ranavirus susceptibility 2) larval growth rates and survivorship and 3) viral loads.

CHAPTER 2

PREVALENCE OF AMPHIBIAN PATHOGENS IN LARVAL AMBYSTOMATID SALAMANDERS[1]

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ABSTRACT

A variety of biotic and abiotic stressors have been hypothesized to increase the emergence of disease in amphibians by increasing host susceptibility. A wetland's hydroperiod can influence species composition and larval period. However, few studies to date have examined the linkages between natural hydroperiods and presence of pathogens in amphibians, and this knowledge gap impedes our understanding of pathogen transmission in both disturbed and natural systems. *Batrachochytrium dendrobatidis* (Bd) and ranaviruses are amphibian pathogens currently infecting amphibians throughout the world. Bd has caused amphibian population die-offs throughout North and Central America and ranaviruses are causing population fluctuations in herpetofauna in the Southeastern United States. In order to understand the important of hydroperiod variation in amphibian pathogen dynamics, I surveyed two Ambystomatid species for both Bd and ranavirus in 20 wetlands on the Savannah River Site, SC, USA in 2014 (N=464) and 2015 (N=720). We only detected ranavirus in 3 larval marbled salamanders in 2014, and no presence of either disease in 2015. This study provides multi-year information for locations where both pathogens were previously recorded that now have no presence of the pathogen in the populations tested. This highlights the importance of long-term surveillance studies, but also the importance of studying cold-spots in amphibian conservation.

Key Words: Amphibian; *Ambystoma*; *Batrachochytrium dendrobatidis* (Bd); Hydroperiod; Ranavirus; Salamander

INTRODUCTION

Multiple factors are considered to contribute to the worldwide decline of amphibians, including habitat destruction and alterations, climate change, invasive species, and emerging infectious diseases (Carey 1993; Daszak et al. 1999; Kiesecker and Skelly 2001). It is unlikely that any single factor is solely responsible for the increased declines, as it is rare that amphibians experience a single factor at any given time (Blaustein and Kiesecker 2002; Blaustein et al. 2011). One hypothesis for recent declines is that environmental pollution and other anthropogenic stressors have increased susceptibility to pathogens by impairing immune function (Daszak et al. 1999). Two pathogens, ranaviruses and *Batrachochytrium dendrobatidis* (Bd), are commonly the focus of controlled laboratory experiments and field-based prevalence surveys due to their widespread distribution (Daszak et al. 1999) and implications on population-level declines (Jancovich et al. 1997; Berger et al. 1998; Lips 1999; Teacher et al. 2010; Price et al. 2014). How these two pathogens interact with wetland drying regimes on the landscape is poorly understood but necessary for the understanding of pathogen dynamics.

Various ranaviruses infect ectothermic hosts, like amphibians, reptiles, and fish, and are implicated in amphibian die-off events around the world (Price et al. 2014). In amphibians, ranavirus infection and disease are strongly associated with life-history stage, with most observed die-offs occurring in late larval development (Speare and Smith 1992; Green et al. 2002; Green and Converse 2005; Greer et al. 2005; Warne et al. 2011). Thus, factors affecting the duration of larval life stages (i.e., species-specific development time or plastic responses to environmental conditions) may also influence

the amount of time a larva is exposed to a pathogen and the infection intensity of the pathogen itself. For instance, wetland temperature significantly affects amphibian time to metamorphosis (Harkey and Semlitsch 1988; Dimauro and Hunter 2002; Loman 2002; Vignoli et al. 2007), and permanent wetlands can often be colder than wetlands with shorter hydroperiods (Wilbur 1987). Ranavirus prevalence also varies between host populations, species infected, and seasons (Greer et al. 2009; Hoverman et al. 2012; Rothermel et al. 2013). For example, tiger salamander (*Ambystoma tigrinum*) populations in Arizona, U.S. varied greatly (0-57%) in overall prevalence of ATV (*Ambystoma tigrinum* virus), a species of *Ranavirus* (Greer et al. 2009). Presence can also vary by species, for example, the proportion of stream salamanders sampled that were positive for ranavirus varied from 10-50% in 7 different species (Rothermel et al. 2013). Additionally, Hoverman et al. (2012) observed infection across multiple seasons with 61% prevalence in wetlands studied and 63% of species sampled at each site. Given the variance in prevalence found in previous studies, it is important to study multiple aspects of the habitat when surveying for ranavirus, particularly in diverse ecosystems.

Batrachochytrium dendrobatidis (Bd), a fungal spore that causes chytridiomycosis, is a generalist pathogen, infecting multiple genera of amphibians throughout the world (Berger et al. 1998; Lips et al. 2006; Olson et al. 2013). Resistance to the fungus can also vary between amphibian species due to differences in innate immune defenses (Woodhams et al. 2007). Bd is well-studied in amphibians that are currently in decline, of conservation concern, or in areas where other species are experiencing mortality events (Berger et al. 1998; Bosch et al. 2001; Lips et al. 2006). Many factors such as macroinvertebrate community composition (Strauss and Smith

2013), habitat structure (Becker and Zamudio 2011; Savage et al. 2015), temperature (Berger et al. 2004), amphibian host diversity (Searle et al. 2011) and density (Rachowicz and Briggs 2007) can influence Bd dynamics. Many studies document Bd in areas without declines or mortality in common amphibian species (Peterson et al. 2007; Hossack et al. 2010; Richards-Hrdlicka et al. 2013; Korfel and Hetherington 2014). These complex interactions offers great opportunity to understand drivers of pathogen prevalence, and highlights the importance of research focused on species or habitats that are not currently experiencing mortality events and observations (i.e. cold spots; James et al. 2015). In order to understand how important community composition, habitat structure, and other environmental factors are, we must understand pathogen prevalence across the entire landscape, not only in areas of known mortality, but also in areas without previous research.

A wetland's hydroperiod, or the total amount of time water remains in a wetland (Ryan and Winne 2001), can influence many factors linked to pathogen prevalence. Amphibian larval period can vary with hydroperiod length (Pechmann et al. 1989; Denver et al. 1998; Morey and Reznick 2000; Snodgrass et al. 2000a). Additionally, amphibian (Pechmann et al. 1989; Snodgrass et al. 2000b; Semlitsch et al. 2015) and macroinvertebrate (Zokan and Drake 2015) species richness is related to wetland hydroperiod length. Factors such as pH, dissolved oxygen, and temperature can vary with hydroperiod as well (Dimauro and Hunter 2002; Lindquist et al. 2013; Schriever et al. 2014). Consequently, hydroperiod length may be an important factor affecting pathogen prevalence indirectly through its impacts on numerous biotic and abiotic factors. Yet, few studies have compared prevalence across wetlands with varying hydroperiods and instead

have focused on permanent wetlands (e.g. Strauss & Smith 2013) or species found in semi-permanent wetlands (e.g. Rojas et al. 2005, Brunner et al. 2007, Homan et al. 2013, Earl & Gray 2014, North et al. 2015). To investigate the importance of hydroperiod on amphibian disease dynamics, I investigated pathogen presence and prevalence in two Ambystomatid salamander speciesacross wetlands of differing hydroperiod.

METHODS

Study Sites

The United States Department of Energy's Savannah River Site (SRS) near Aiken, SC encompasses 780 km^2. The SRS is designated as a National Environmental Research Park, and contains critical wildlife habitat as less than 95% of the SRS is directly impacted by site operations. On the SRS there are over 30 species of pond-breeding amphibians in over 100 wetlands (Gibbons and Semlitsch 1991; Burger and Snodgrass 2000). We included 20 wetlands that occur along a hydrologic gradient based on long-term data from the University of Georgia Savannah River Ecology Lab (SREL). However, there is overlap in wetland type during exceptionally wet or dry years. Thus, species that typically are considered short or long hydroperiod specialists may occur in the same wetland at times.

We focused our study on *Ambystoma talpoideum* (mole salamander) and *Ambystoma opacum* (marbled salamander) because they are closely related, similar physiologically, and share a similar ecological niche. Mole salamanders have longer larval developmental periods (4 to 15 months) and are commonly found in longer hydroperiod wetlands. Marbled salamanders specialize in short hydroperiod wetlands

where they lay eggs terrestrially, that hatch when the wetland fills, and then have a rapid development (3-6 months). Thus, marbled salamanders are limited to wetlands that dry regularly. Both species co-occur in wetlands that have intermediate hydroperiod lengths and mole salamanders can occur in shorter hydroperiod wetlands where recruitment only occurs in wet years.

Larval amphibians were captured in minnow traps or by dipnetting from 22 March – 4 May, 2014, and from 8 March – 22 June, 2015. Individuals were placed in Ziploc® bags with water from the wetland, and brought back to the lab. Once in the lab, we swabbed and weighed individuals, then euthanized in MS-222 prior to dissection for liver and tail tissue. We recorded liver weight, snout-vent length (SVL), and total length for each individual. All samples except for liver tissue and swabs were stored in 70% ethanol and placed in a -20°C freezer. Liver tissue was stored in a -80°C freezer to maintain for future culturing of the virus. Swabs were stored in the -20°C freezer. All methods were approved by the University of Georgia's IACUC – 06-013-A0.

DNA Extraction

We followed the DNeasy Blood and Tissue Kit (Qiagen©, Valencia, CA) to extract DNA from liver and tail samples for ranaviral testing and Bd testing respectively. Each sample was eluted using 200 μL of buffer. We determined DNA concentrations using a NanoDrop (Nanodrop Spectophotometer ND-1000, Thermo Scientific).

Ranavirus

To test for ranaviral infections, we used quantitative PCR (qPCR) following the thermal profile protocol and primer set from Allender et al. (2013). Each sample was run in triplicate on the same plate with a negative control and a serial dilution of positive

standard from 10 to 10^6 viral copies in order to calculate viral load on each individual. The positive standard was isolated from a northern leopard frog (*Lithobates pipiens*). Each standard was replicated on the plate at least 3 times with the 10^2 and the 10^1 standards replicated 4 and 5 times respectively. No positive samples were recorded if the threshold cycle value was higher than for the lowest standard on the plate.

Bd

We also used qPCR to detect Bd infections by using tail tissue from each individual. We utilized the fast qPCR methods and reaction volume from Kerby et al. (2013) and primers developed by Boyle et al. (2004). We ran samples in triplicate with a serial dilution of positive standard from 0.1 to 100 viral copies as well as a negative control. We used a local isolate (SRS810; isolated from a bullfrog *Lithobates catesbeianus* on the SRS) as the standard for each plate to create the standard curve. The standards were replicated 5 times on each plate to generate an accurate standard curve. Again, no positive samples were recorded if the threshold cycle value was higher than that for the lowest standard value on the plate.

Statistical Analysis

We used the standard curve generated by each plate to determine viral copy estimates.

RESULTS

In 2014, we analyzed 464 individuals for ranavirus and Bd infection over a three month period from March 22, 2014 to May 4, 2014. There was no Bd detected in either mole (N=321) or marbled (N=142) salamanders. In 2014, we detected three individuals positive for ranaviral infection at one location, Bay 128, in a single species, marbled

salamanders. We analyzed 720 salamanders (marbled N=275; mole N=437) for both ranavirus and Bd infection from March 8, 2015 to August 25, 2015 at 25 wetlands. We did not detect either pathogen in any marbled or mole salamanders during 2015.

DISCUSSION

We did not find any individuals positive for Bd in this study. Previous studies on the SRS did find Bd in amphibians, including museum specimens from 1978 and 1981 (Daszak et al. 2005), individuals at contaminated wetlands (Peterson et al. 2007), and in 2011-2012 (Love et al., *in review*). Peterson et al. (2007) recorded 64% prevalence in a wetland system with a history of copper and mercury contamination. Unlike our study, this study focused exclusively on anurans and did not include salamanders. Additionally, a recent study observed Bd prevalence ranging within a species from 0-48%, and a total prevalence of 9.7% across the SRS in 2011-2012 (Love et al., *in review*). Mole and marbled salamanders had an overall prevalence of 3% for Bd in that study, which was low compared to anurans, but was the first documentation of Bd in salamanders on the SRS. Bd prevalence can be seasonal and found primarily in adults, our study consists only of larval individuals, potentially not capturing the peak of Bd prevalence in salamander populations on the SRS. This highlights the annual cycles Bd can have in amphibian populations and the importance of extending surveillance into long-term monitoring studies.

Additionally, we only detected ranavirus in three marbled salamanders out of 464 total salamanders sampled. In Love et al. (*in review*), mole and marbled salamanders had the greatest infection of ranavirus of salamander species, 52.5% and 45.9% respectively.

A mortality event attributed to ranaviral infection also recently occurred on the SRS in an eastern mud turtle (*Kinosternon subrubrum*; Winzeler et al. 2015). Therefore, it is surprising that we detected such low levels of ranavirus in mole and marbled salamanders on the SRS. Ranavirus can be seasonal, but also cause increased viral prevalence and mortality in annual cycles (Teacher et al. 2010), resulting in annual fluctuations within the same wetland. This study could be documenting years of extremely low prevalence of ranavirus in larval salamanders between high prevalence and infection events. Other studies of ranavirus in the Southeast document ranavirus infections across multiple seasons in wetlands with long hydroperiods around 60% prevalence (Hoverman et al. 2012) but do not include wetlands with a shorter hydroperiod.

Our study provides valuable, multi-year information about the prevalence of Bd and ranavirus on the SRS, and highlights the need for long-term data in areas considered low priority for die-off events. These systems can provide valuable knowledge of the dynamics and evolution of host-pathogen relationships in systems when die-off events are irregular and prevalence is seasonal and changes annually. Recently there was a call to study "cold spots" for amphibian pathogens instead of focusing in areas with mortality events (James et al. 2015). Our study adds to the growing body of literature that shows fluctuations in natural host-pathogen dynamics, which can be critical to future conservation of amphibian populations.

CHAPTER 3

EFFECTS OF EARLY EXPOSURE TO MULTIPLE STRESSORS ON LARVAL AMPHIBIAN PATHOGEN SUSCEPTIBILITY[2]

[2] Winzeler, M. E., and S. L. Lance. To be submitted to *EcoHealth*.

ABSTRACT

Amphibians experience multiple stressors during their development, and the effects of these stressors include decreased survival, lengthened larval periods, and other physiologic changes. How these stressors impact amphibians exposed to pathogens is less understood. In this study we experimentally manipulated two environmental stressors (Cu and hydroperiod), and examined their effects on the susceptibility of larval marbled salamanders (*Ambystoma opacum*) to a FV3-like ranavirus. We reared individual larvae in a 2x2x2 factorial design, with treatments including: exposure to Cu (0 and 30 μg/L), hydroperiod length (none or periodic removal of water), and exposure to a FV3-like ranavirus (sham or 1.71×10^5 PFU/mL). The Cu and hydroperiod treatments began upon hatching, while the ranavirus exposure occurred at 30 days post hatching. We measured survival and total length on days 0, 30, and 60 to calculate growth rates and quantified viral load of ranavirus using qPCR at day 60. We used ANOVA and Tukey's HSD to determine differences between treatment and exposure group (unexposed - U, exposed and negative – E-, exposed and positive – E+) in total length, growth rates, survival, and viral load. Of individuals exposed to the virus (N=120), 24 were negative for ranavirus at the end of 60 days. There was no significant effect of treatment on either total length, growth rate, or viral load. There was a significant effect of exposure group on total length on day 60: E+ were significantly smaller than E-, U were not significantly different from either the E- or E+ groups. Growth rate varied significantly among exposure groups and was the greatest in the E- group. Individuals E+ had the lowest growth rates, with E- and U growth rates not significantly different from each other. Individuals with the highest growth rates had the lowest viral load estimates as well. Although survival was not

affected, our study highlights the importance of studies on the sublethal impacts of exposure to multiple stressors and costs of pathogen infection in amphibian populations.

Key Words: Amphibian; *Ambystoma opacum*; Copper; Hydroperiod; Marbled salamander; Ranavirus; Sublethal

INTRODUCTION

Wildlife stressors include biotic and abiotic aspects of the environment that can cause a physiological change in an individual (Johnstone et al. 2012)and can be natural, like predation (Reeves et al. 2011), wetland drying (Amburgey et al. 2012), and competition (Atobe et al. 2014), or human-caused, like heavy metal contamination (Hopkins et al. 1997; Ficken and Byrne 2013) and habitat fragmentation (Cushman 2006). Exposure to a single stressor can influence the health of wildlife (Boone and Bridges 2003; Albert et al. 2007; Lavergne et al. 2014; McArthur et al. 2014) but since they rarely experience one stressor at a time it is necessary to understand the interactive effects of multiple stressors on wildlife. When multiple stressors are present, a change in the duration or concentration of one stressor can greatly influence the effect of a second stressor on the individual (Relyea and Mills, 2001; Chen et al., 2004; Rohr et al., 2004). For example, when gray treefrogs (*Hyla versicolor*) were exposed carbaryl, a common pesticide, the pesticide was up to 4 times more lethal when combined with predator cues (Relyea and Mills 2001). Examining how the combination of stressors and their interactions affects individuals is critical to understanding their potential influences on the persistence of wildlife populations.

One class of stressors increasingly studied due to their implications in population and community regulation are amphibian pathogens (Daszak et al. 2003a). In particular, ranaviruses (Family: Iridoviridae) and the fungus *Batrachochytrium dendrobatidis* (Bd) are emerging pathogens implicated in amphibian declines throughout the world (Carey and Alexander 2003; Daszak et al. 2003a; Price et al. 2014). One hypothesis is that the sudden emergence of amphibian pathogens is partly due to a weakened host immune

response resulting from exposure to environmental contaminants (Christin et al. 2003; Rohr et al. 2008). For example, exposure to atrazine and chlorpyrifos increased susceptibility to a ranavirus (ATV) in tiger salamanders and decreased survival of early stage larval (*Ambystoma tigrinum*; Kerby and Storfer 2009). However, larval anurans exposed to both a predator and FV3-like ranavirus stressors did not experience increased mortality or viral load (Haislip et al. 2012) indicating that the interactive effects of stressors are not consistent. It is necessary to study the interactions between amphibian pathogens and other biotic and abiotic stressors to fully understand their combined impacts on amphibian populations.

The amount of time a wetland holds water, its hydroperiod (Ryan and Winne 2001), is an important abiotic stressor and critical to successful larval amphibian development because it can affect time to metamorphosis and overall survival (Richter-Boix et al. 2011). Pond-breeding amphibians vary in their hydroperiod requirements with some species only found in relatively short hydroperiod wetlands (Snodgrass et al. 2000b). These short hydroperiod wetlands are at a greater risk of early drying, increasing desiccation risk (Pechmann et al. 1989), than wetlands with longer hydroperiods. Accelerated pond drying can lead to early emergence (Koprivnikar et al. 2014), lower mass and shorter limbs (Gomez-Mestre et al. 2013), or even catastrophic reproductive failure when the wetland dries before any larvae successfully metamorphose (Taylor et al. 2006). While responding physiologically to current hydrologic conditions can be beneficial in many cases (i.e. escape predation or poor water quality conditions; Hamer and Parris 2013), limited energy resources during early life stages can affect later life stages, like reduced mass and elevated stress hormones (Crespi and Warne 2013).

Another common stressor for amphibians is exposure to metal contamination (Fedorenkova et al. 2012) that can have strong lethal and sublethal impacts on amphibian larval development (Willson et al., 2012; Lance et al., 2012, 2013). Copper (Cu) is a common by-product of pesticide and fertilizer use, coal fly ash, brake wear, and other industrial products (USEPA 2009). In an ecological risk assessment of common environmental stressors, Cu was named the second highest stressor risk to amphibian populations in Europe (Fedorenkova et al. 2012). When exposed to Cu, embryos of the South American common toad (*Rhinella arenarum*) experienced sublethal effects including delayed development, reduced body size, and under-developed gills (Aronzon et al. 2011). Similarly, exposure to Cu increased larval period in southern leopard frogs (*Lithobates sphenocephalus*) (Lance et al. 2012) and delayed development in the eastern narrowmouth toad (*Gastrophyrne carolinensis*) (Flynn et al. 2015). The impacts of sublethal exposure to amphibian populations could negatively impact long-term population dynamics (Carey and Bryant 1995; Salice et al. 2011; Salice 2012). Sublethal effects of Cu such as these are well studied in single-stressor studies, but the influence of Cu when combined with other stressors, like shortened hydroperiod or a pathogen, are poorly understood.

Host-pathogen dynamics are well-studied in many wildlife species, including complex interactions with multiple stressors (Hudson and Greenman 1998; Begon et al. 2003; Garbutt et al. 2014). Many studies on amphibians aim to quantify the costs of parasite infection and load (Tocque 1993; Johnson et al. 2006; Johnson et al. 2012). Few studies focus on how exposure to an early life stage stressor, like metals contamination or fluctuating hydroperiod, impacts later development and fitness. Our objective was to

determine how the exposure to multiple stressors (FV3-like ranavirus, Cu, and shortened hydroperiod affected larval marbled salamanders (*Ambystoma opacum*) when experienced alone and in combination. I predicted that exposure to single stressors would have the following outcomes: both Cu and ranavirus causing reduce survival and/or growth rate and a shortened hydroperiod causing accelerated growth. When experienced in combination I predicted antagonistic effects on growth from exposure to Cu and a shortened hydroperiod treatment. I expect that ranavirus will negatively affect survival and growth in all individuals exposed to the virus. I also predicted antagonistic effects on growth and survival when exposed to ranavirus after exposure to Cu and shortened hydroperiod.

METHODS

Experimental Design

In the fall of 2014, I collected adult marbled salamanders from a Carolina bay (Rainbow Bay) on the Department of Energy's Savannah River Site (SRS) near Aiken, SC. I housed multiple breeding pairs of marbled salamanders in four outdoor mesocosms (0.6 x 1.8 m galvanized stock tank, 1476.3 L). Mesocosms included woody debris, 5-7 sedges (*Carex* spp.), and soil substrate with a shade-cloth cover allowing permeability of weather conditions. I collected 10 egg masses on 3 February 2015, mixed the clutches together, and inundated them with soft water (48 mg/L $NaHCO_2$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, and 2 mg/L KCl at added to 50 L of nanapore Milli-Q® water) overnight to induce hatching. Upon hatching, I moved 240 larvae into individual plastic containers

(9.6 cm diameter, 10.9 in. H, 0.5 L, Berry Plastics Corporation, Evansville, IN, USA) with 1 L of soft water.

I used a 2x2x2 factorial design with two levels of Cu exposure (0 and 30 µg/L; N=30), two levels of hydroperiod (long and short; N=30), and two levels of pathogen exposure (no virus or virus; N=30) with 30 replicates of each treatment combination. The hydroperiod treatment was applied for the first 30 days of the experiment, the Cu treatment for all 60 days, and a 24 hour ranavirus exposure took place on day 30. Based on previous research marbled salamanders have high post hatchling survival in Cu levels below 50 µg/L Cu, (Soteropoulos et al. 2014), therefore I used 30 µg/L Cu as a sublethal exposure. For pathogen exposure, I used a strain of ranavirus that I isolated and cultured from an infected wild *A. opacum* caught in 2014 on the SRS and following the protocols described by Ariel et al. (2009) using a fathead minnow cell line. I created the shortened hydroperiod treatment by removing 200 mL of water weekly for the first 30 days of the experiment for a total of 600 mL removed. While other factors may influence how amphibians respond to pond drying, such as density increases and nutrient fluctuations (Harkey and Semlitsch 1988; Tejedo and Reques 1994), I chose to focus on reduced water volume. For the Cu exposure treatment, I dosed containers with 0 or 30 µg/L solution of $CuSO_4$ in each experiment container.

On day 30, I moved all individuals to new 50mL containers containing soft water and added 500 µL of either 1.71 x 10^5 PFU/mL FV3-like ranavirus (pathogen exposure; N = 120) or Minimum Essential Medium with Hank's salts (sham exposure; N=120, 30/treatment). This virus concentration is similar to other exposure studies (Brunner et al. 2007; Johnson and Brunner 2014; Grayfer et al. 2014), and I cultured a local strain for

exposure to minimize a lethal exposure to a novel virus. After 24 hours of exposure, I moved individuals back into their original treatment containers. After day 31, water changes continued weekly to maintain water quality and Cu levels; however, the drawdown of hydroperiod treatments ceased. Throughout the experiment, I checked all individuals daily. On days 1, 30, and 60 I photographed all individuals from a standardized height with a ruler (mm) in the image and later analyzed them using ImageJ (Schneider et al. 2012) to calculate total length. When an individual died I removed it, measured its total length and took tail and liver tissue for DNA analysis (see below). From day 0 to day 17 I provided *Daphnia ad libitum* and from that day until Day 60 I provided blood worms *ad libitum*.

Pathogen presence testing

On day 60 of the experiment I euthanized all larvae by submersion in MS-222 solution and placed whole bodies in 70% ethanol-filled 1.5 µL centrifuge tubes which were stored in a -20°C freezer until later analysis. To perform DNA extractions, I lysed a combination of tail tissue and liver tissue from each salamander larvae with a DNeasy Blood and Tissue Kit (Qiagen©, Valencia, CA) and following manufacturer's protocol with the exception that the final elution volume was 100µL. I determined DNA concentrations using a NanoDrop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA, USA).

To test for ranaviral infections, I used quantitative PCR (qPCR) following the thermal profile protocol and primers described in Allender et al. (2013) to test for pathogen presence and total viral load. Briefly, the reaction consisted of 1x TaqMan Platinum PCR Supermix-UDG ROX (TaqMan® Platinum PCR Supermix-UDG with

ROX, Invitrogen, Carlsbad, CA) with ROX, 2x TaqMan primer-probe, 2.5 μL of DNA, and water to a final volume of 12.5 μL. I ran each sample in triplicate on the same plate with a negative control and a serial dilution of positive standard from 10 to 10^6 viral copies/μL in order to calculate viral load on each individual. I replicated standards on the plate at least 3 times with the 10^2 and the 10^1 standards replicated 5 times. I determined samples to be positive for infection if the threshold cycle value was lower than the C_t value of the lowest standard on the plate for at least two of the three replicates. I quantified viral loads for positive individuals using the standard curves generated from the individual's plates. I only maintained data from plates with a PCR efficiency above 90%. All methods were approved under the University of Georgia's IACUC – 06-013-A0.

Statistical Analysis

For the purposes of some statistical analyses I separated the ranavirus exposure group into those that were unexposed (U), those exposed but testing negative (VE-) and those exposed and testing positive (VE+). To determine differences of survival by treatment and exposure group I used a Cox Proportional Hazards model. I used an analysis of variance (ANOVA) to determine difference treatment and pathogen exposure group on total length. A Tukey's honest significant difference (HSD) test to determine differences between these groups when there was significance. I assessed assumptions using Shapiro-Wilk for normality and Bartlett's Test for homoscedasticity and viral loads data violated both assumptions. I corrected this by using a log transformation for the total viral loads data only. I performed a multiple regression that included total length at day 60 and viral

load of the individual. I performed all analyses using R statistical software (Version 3.1.0).

RESULTS

Ranavirus Exposure

The sham exposed treatment group was 100% negative for infection. Of individuals exposed to the virus, 83.4% tested positive across all Cu/hydroperiod treatments. Infection prevalence varied within Cu/hydroperiod treatment but was not significant; 83.3% in the shortened hydroperiod and 0µg/L Cu, 86.6% in the 30 µg/L Cu treatment, and 80.0% in the combined Cu and shortened hydroperiod treatment.

Survival

Overall, 208 of 240 individuals survived to day 60 with the highest survival in the control group (100%) and the lowest in the virus exposed, 30 µg/L Cu and shortened hydroperiod group (81.6%). There was no significant difference in survival due to exposure to only Cu and/or a shortened hydroperiod (Table 1; Figure 1). When only considering the virus exposure groups survival was significantly decreased in the VE- exposure group. (Table 1; Figure 2).

Total Length

By chance, on day 0 the group assigned to ranavirus exposure was significantly larger than the sham exposure group (Table 2) but there was no significant difference among those assigned to the different Cu and hydroperiod treatments (Table 2). By day 30, there was a significant difference by Cu/hydroperiod treatments (Table 2) but there was no longer a difference between the groups assigned to ranavirus and sham exposures

(Table 2). There were no differences between any combination of Cu/hydroperiod treatments besides the shortened hydroperiod and combined shortened hydroperiod and Cu treatments (Table 2), with shortened hydroperiod individuals larger than shortened hydroperiod and Cu individuals. Finally, on day 60, there was no significant effect of Cu/hydroperiod treatment or pathogen treatment (Table 2). However, total length did differ by pathogen exposure group on day 60 (Table 2; Figure 3). Individuals testing positive for ranavirus (VE+) were significantly smaller than VE- individuals (Table 2; Figure 3). The unexposed individuals (U) were not significantly different from either the VE- or the VE+ groups (Table 2; Figure 3).

Viral load

Viral load ranged from 0.03 to 53,590 copies per µL of extracted DNA with an average viral load of 537 copies. There was no significant effect of the Cu or shortened hydroperiod treatments on viral load (Table 2) in individuals that tested positive for ranavirus. Total length was significantly affected by viral load on Day 60 (Table 2), with smaller individuals having higher viral loads (Figure 5).

DISCUSSION

Copper and hydroperiod stressors did not affect larval marbled salamanders' growth rates, survival, or susceptibility to ranavirus. In the first 30 days of the study, individuals experiencing shortened hydroperiod and the combined Cu and shortened hydroperiod were significantly larger in total length with no other differences between treatments, but this effect was gone by day 60. At the end of the experiment, the individuals testing positive for ranavirus were the smallest. Only size differed between shortened hydroperiod alone and in combination with Cu but this effect is gone at the end

of the 60 day experiment. This result is surprising due to the number of studies that have demonstrated how influential hydroperiod is during the development of larval amphibians (Wilbur and Collins 1973; Wilbur 1987; Semlitsch and Wilbur 1988; Denver et al. 1998; Loman and Claesson 2003). Our water reduction treatment might not have been severe enough, created the right cues, or over a long enough period to induce a stress response during later development. Amburgey et al. (2012) reduced water volume through metamorphosis of the larval amphibian but did not see any effects of simulated shortened hydroperiod. Pacific chorus frogs (*Pseudacris regilla*) in short hydroperiod treatments emerged earlier and were more developed than tadpoles not experiencing water reduction (Koprivnikar et al. 2014). However, if another factor, like temperature or density, prompts metamorphosis our study design would not allow us to detect those changes because I focus solely on hydroperiod length. Tadpoles in low larval densities that experienced simulated pond drying had shorter larval periods than in the long hydroperiod treatment (Tejedo and Reques 1994). Increased temperatures also caused decreased time to metamorphosis and size at metamorphosis in ornate chorus frogs (*Pseudacris ornata*; Harkey and Semlitsch 1988). Multiple factors can contribute to cue amphibian metamorphosis and future studies should focus on a combination of cues, rather than a single cue.

The effects of heavy metals on amphibians include decreased survival, altered developmental times, and reduced body size (Chen et al. 2007; Peles 2013; Flynn et al. 2015). I did not see any effect of the Cu treatments or the combined interaction of Cu and reduced hydroperiod on survival, total length or growth rate. I used a concentration of Cu (30 µg/L) found to reduce survival on larval southern toads (*Anaxyrus terrestris*) (Lance

et al. 2013) and larval eastern narrowmouth toads (Flynn et al. 2015). However, that level of Cu did not affect embryo survival of marbled salamanders suggesting they may be more tolerant (Soteropoulos et al. 2014). Positive individuals within treatments was highest in the Cu-only treatment (86.6%) but lowest in the Cu/hydroperiod treatment (80.0%) but there was no significant effect of either stressor on susceptibility to ranavirus. In gray treefrog (*Hyla chrysoscelis*), Cu exposure did not affect time to metamorphosis or larval growth but did slow development when in combination with the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) (Parris and Baud 2004). Since I did not see an on Cu on ranavirus susceptibility, there might be differences in how Cu affects fungal pathogens versus viral pathogens or species-specific differences in Cu effects.

Unlike the Cu and hydroperiod treatments, exposure to ranavirus significantly affected growth rates. The VE+ group experienced decreased growth rates that could be due to a reallocation of resources during development to immune defense. The VE- group actually had the fastest growth rates, perhaps suggesting that individuals in better condition have immune responses that are more robust. However, this group also had the lowest overall survival suggesting a potential cost to the accelerated growth. Importantly, because we only tested for ranavirus on Day 60 (or upon death) we cannot be sure that the VE- survivors had successfully fought off an infection or never had an infection. Given that infected individuals with lower viral loads had higher growth rates and total lengths there may indeed be a relationship between growth rates and immune function. The introduction of a pathogen requires some of resources spent towards immune defense and maintenance, evidenced by the relationship between higher viral loads and lower

growth in the exposed and positive group. These findings have important implications for potential sublethal effects of pathogen exposure. Because our study stopped at 60 days I cannot determine whether the ranavirus positive larvae would successfully reach metamorphosis, but the data indicate that time to- and size at-metamorphosis would be affected. However, individuals that were VE- for ranavirus had growth rates similar to individuals that were unexposed to the virus. This could represent individuals with strong immune systems that were never successfully infected, but it could also show individuals increasing growth rates to compensate for previously low rates. Research extended to metamorphosis may better determine how the exposed and negative group performs compared to the other two exposure groups.

Most amphibians have complex life history cycles (Wilbur and Collins 1973) such that an individual can experience a stressor early in life in the aquatic environment that impacts development and health at the terrestrial life stage (Berven 1990; Van Buskirk and Saxer 2001). Stressors such as contaminant exposure and shortened hydroperiods commonly cause sublethal impacts on amphibians (Boone et al. 2005; Roe et al. 2006; Brand et al. 2010; Flynn et al. 2015) and use population models to predict population level impacts (Salice et al. 2011; Zipkin et al. 2012; Salice 2012; Amburgey et al. 2014; Chandler et al. 2015). However, with amphibian pathogens it has been more common to focus on population die-off events (Greer et al. 2005) and not the potential sublethal impacts that can also affect long-term population persistence or population modeling common in host-pathogen systems. Our results indicate that even when exposure to ranavirus does not result in morbidity or mortality; it may still have significant impacts. The reduced growth rate I see in infected individuals may impact

fitness either through an inability to metamorphose before pond drying (enhancing catastrophic reproductive failure) or reduced size at metamorphosis—a correlate for future survival and successful reproduction (Scott 1994; Willson et al. 2012). A recent call for the identification of pathogen "cold spots" and decreased focused on "hot spots" (James et al. 2015) should guide future amphibian pathogen research. Particularly in areas where die-off events are not observed, studying population-level impacts of pathogens, like ranaviruses, is important to understanding host-pathogen dynamics and population vital rates.

Table 1. Results of the Cox-proportional Hazards Model.

Factor	Hazard Ratio (SE)	p-value	95% CI	
			Lower	Upper
Cu (30µg/L)	1.71 (0.36)	0.142	0.835	3.496
Shortened hydroperiod	1.69 (0.36)	0.151	0.826	3.457
Ranavirus sham exposed	0.43 (0.38)	0.027*	0.204	0.911
Ranavirus exposed-negative	3.01 (0.65)	0.093	0.831	10.974
Ranavirus exposed-positive	2.26 (0.39)	0.037*	1.051	4.861

Table 2. Comparison of the repeated measures ANOVA and multi-way ANOVA results.

Model	Df	Mean Sum Sq	F value	P-value
Total Length Day 00				
Treatment (Cu, Hydroperiod, & Interaction)	3	0.0054	0.158	0.924
Virus (Yes or No)	1	0.6835	19.947	<0.001*
Total Length Day 30				
Treatment (Cu, Hydroperiod, & Interaction)	3	0.0935	2.725	0.045
Virus (Yes or No)	1	0.0319	0.930	0.336
Tukey's HSD:		**Lower CI**	**Upper CI**	
Copper (30 µg/L) - Control		-0.112	0.063	0.88
CopperHydroperiod (30 µg/L:shortened) -Control		-0.137	0.038	0.47
Hydroperiod (shortened) - Control		-0.044	0.132	0.56
CopperHydroperiod (30 µg/L:shortened)-Copper (30 µg/L)		-0.113	0.064	0.89
Hydroperiod (shortened) - Copper (30 µg/L)		-0.019	0.157	0.18
Hydroperiod (shortened) - CopperHydroperiod (30 µg/L:shortened)		0.005	0.182	0.03*
Total Length Day 60				
Treatment	3	0.1892	1.193	0.313
Disease (Yes or No)	1	0.3308	2.086	0.150
Final Exposure Group	2	0.6523	4.208	0.016*
Tukey's HSD:				
Unexposed:Exposed-negative				0.240
Unexposed:Exposed-positive				0.102
Exposed-positive:Exposed-negative				0.036*
Viral Loads				
Treatment	3	30,377,419	1.06	0.37
		Std error	**R^2**	
Total length	85	0.3713	0.043	0.051

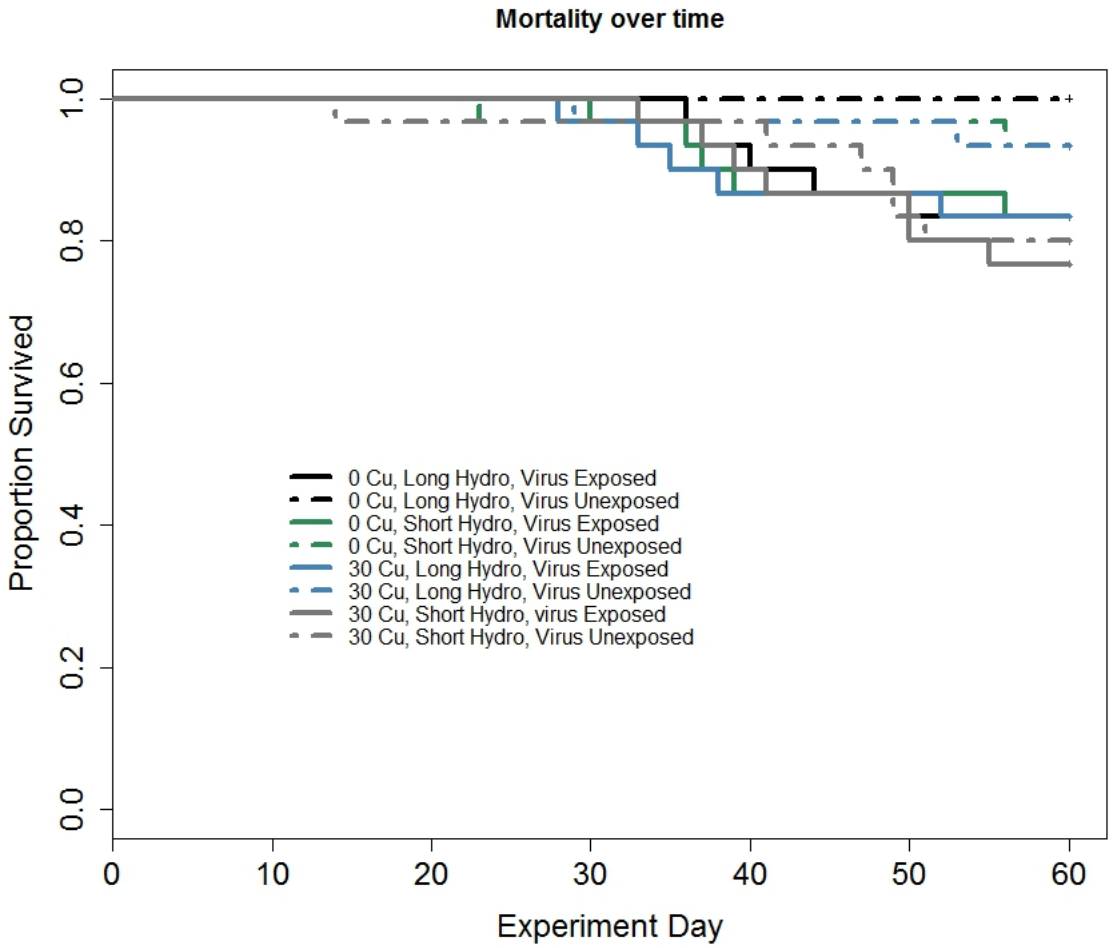

Figure 1. Survival by treatment (control, copper, hydroperiod, and copper-hydroperiod).

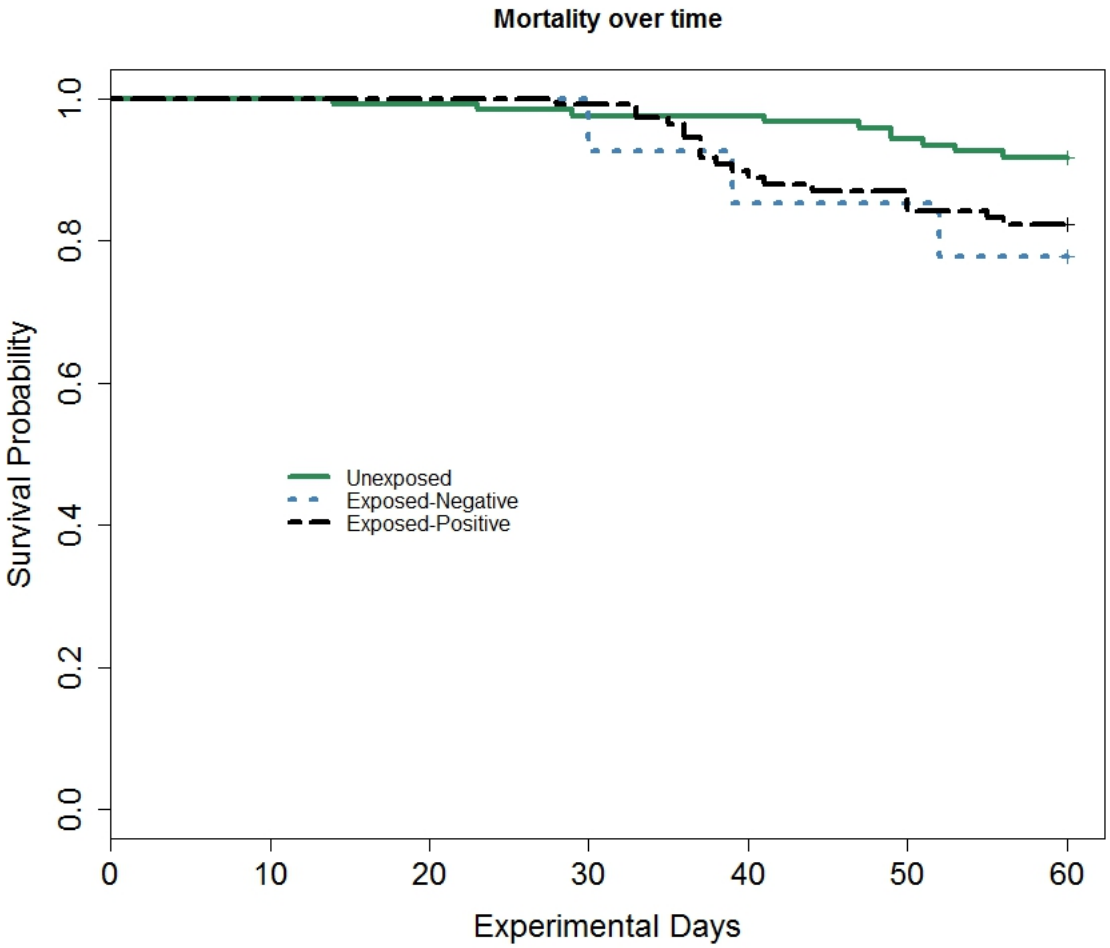

Figure 2. Survival by exposure group (unexposed, exposed-negative, and exposed-positive).

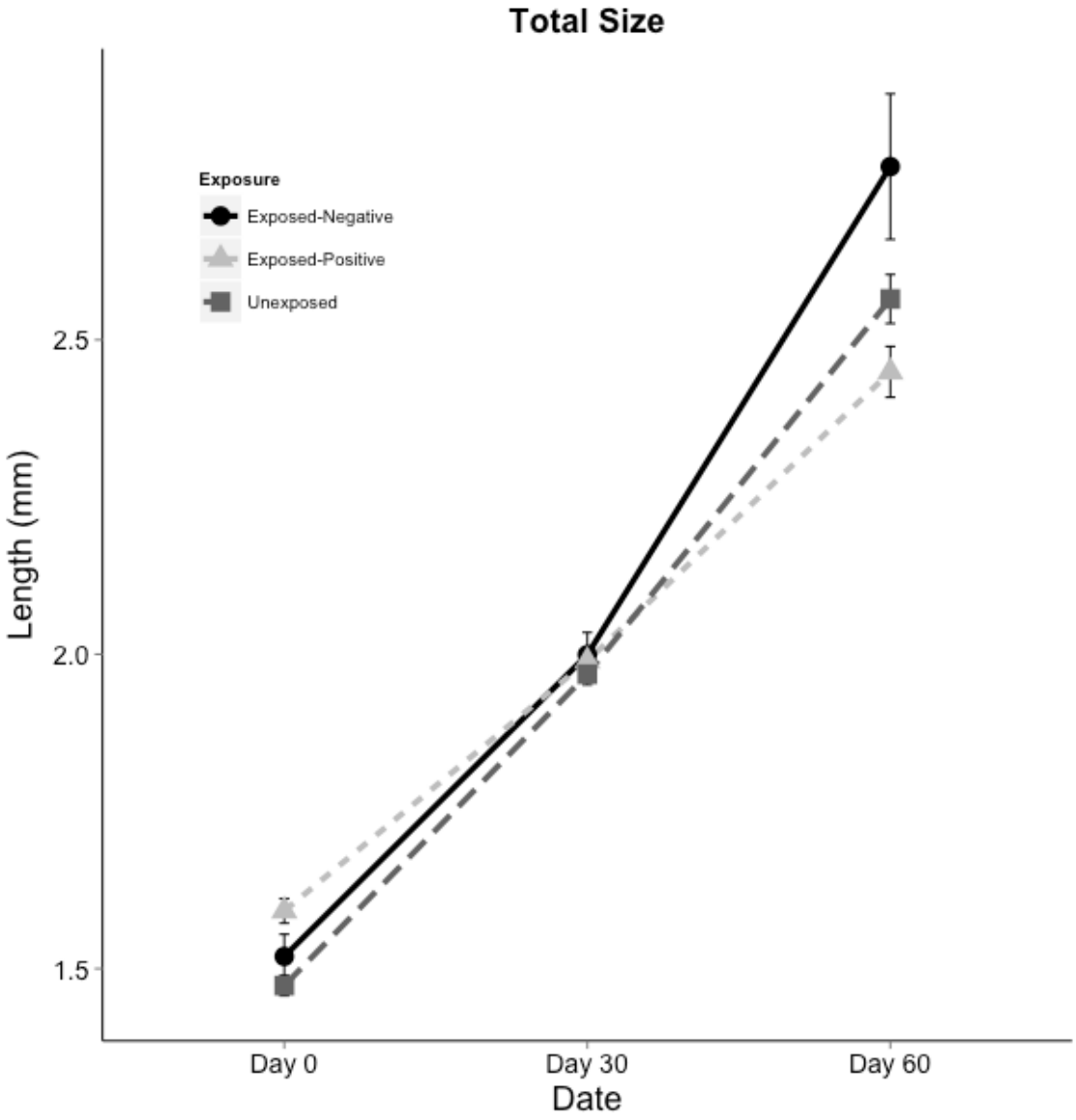

Figure 3. Mean total length of each group (±SE) at days 0, 30, and 60 of the experiment.

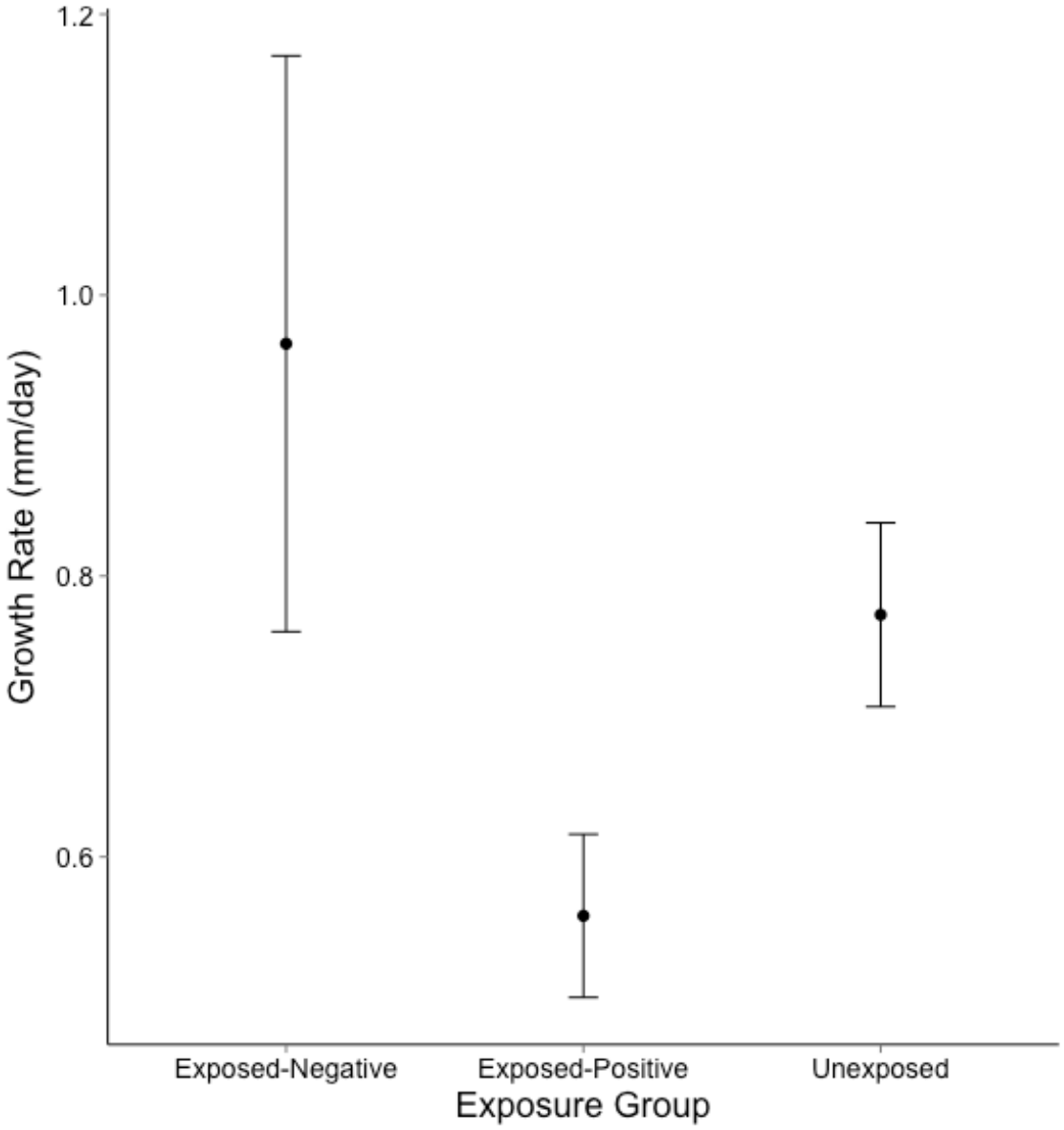

Figure 4. Exposure group (U, E+, and E-) with 95% confidence intervals around the mean growth rate.

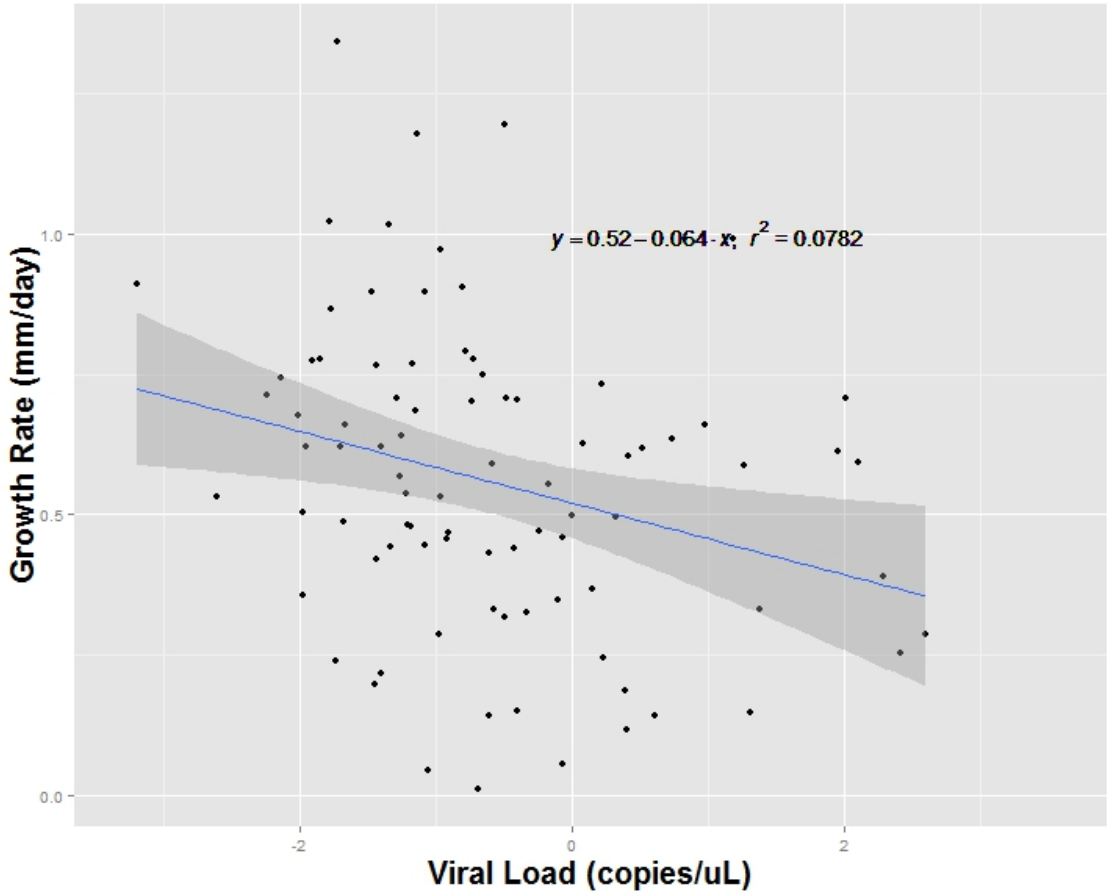

Figure 5. Negative relationship between growth rate and final viral copies (copies/μL). Dark gray shaded area around the regression line is the 95% confidence intervals.

CHAPTER 4

CONCLUSION

As amphibian populations decline worldwide, it becomes increasingly important to understand how biotic and abiotic stressors impact individuals and populations. Exposure to a single stressor can impact amphibian health (Boone and Bridges 2003) but individuals typically experience multiple stressors at a time, potentially altering the effects of a single stressor (Relyea and Mills 2001; Rohr et al. 2004). A group of stressors that are implicated in the decline and extinction of amphibian populations are infectious diseases (Daszak et al. 2003). However, of the interactive effects of exposure to infectious diseases and other stressors in amphibian populations is unclear. Therefore, we aimed to understand how biotic and abiotic stressors impact amphibian populations and their response to pathogen exposure.

Chapter 2 examined prevalence of two pathogens, ranavirus and Batrachochytrium dendrobatidis (Bd) in two Ambystomatid salamander species on the Savannah River Site (SRS). I focused on marbled (*Ambystoma opacum*) and mole (*A. talpoideum*) salamanders because they are closely related but differ in key life history traits. In previous studies on the SRS, both species did have Bd and ranavirus. However, I found low prevalence of both Bd and ranaviruses in two salamander species in all 20 natural wetlands over a two-year time period. I sampled throughout the larval period of both species during the spring months (February – May). Therefore, I might have missed detection of seasonal changes in pathogen prevalence over the course of the study. I also

excluded adults from the survey that might be more likely to be carriers of Bd, but larval periods are ideal to detect ranavirus in amphibians. Few researchers have undertaken large-scale, landscape-level field studies over multiple years to understand amphibian-pathogen patterns. Much of what researchers focus studies on occurs after a die-off event occurred in an area. However, we still understand little of long-term fluctuations in host-pathogen dynamics, particularly for co-infection of multiple pathogens. This study highlights the potential for annual fluctuations in pathogen prevalence and therefore importance of long-term monitoring surveys for amphibian conservation and to understand host-pathogen dynamics.

Chapter 3 focused on an experimental approach to examine the effects of early exposure to multiple stressors on later susceptibility to ranavirus. For the first 30 days, I exposed larvae to one of two levels of copper (0 and 30 g/L) and one of two hydroperiod treatments (static or drawdown) in a fully factorial design. On day 30, I exposed half the larvae in all treatments to a local isolate of ranavirus and observed the effects on growth and survival for an additional 30 days. I did not see any effects of the Cu, hydroperiod, or combination stressors on growth, survival, or susceptibility to ranavirus. There was a significant effect of pathogen presence and viral load on the total length and growth rates of larval marbled salamanders. Growth rates were highest in individuals exposed to ranavirus but negative for the pathogen, less in individuals unexposed, and lowest in individuals exposed to ranavirus and positive for the virus. Within the last group there was also a negative correlation between viral load and growth rate. Reduced growth rates may have significant sublethal impacts on amphibian populations through reduced fitness or extended time to metamorphosis. Thus, pathogen exposure may have important

sublethal impacts on amphibian populations and individual fitness even when die-offs are not observed. This emphasizes the importance of studying the sublethal effects of pathogen exposure to amphibians and their populations.

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APPENDIX A

FIRST CASE OF RANAVIRUS AND ASSOCIATED MORBIDITY AND MORTALITY IN AN EASTERN MUD TURTLE (KINOSTERNON SUBRUBRUM) IN SOUTH CAROLINA[3]

[3] Winzeler, M. E., M. T. Hamilton, T. D. Tuberville, and S. L. Lance. 2015. *Disease of Aquatic Organisms*. 114: 77-81.

ABSTRACT

Ranaviruses are double-stranded DNA viruses that infect amphibians, fish, and reptiles and cause global epidemics in amphibians. It is important to identify new species that may be susceptible to the disease, particularly if they reside in the same habitat as other at-risk species. On the Savannah River Site (SRS) in Aiken, South Carolina, ranaviruses are present within several amphibian populations, but information is lacking on the presence, prevalence, and morbidity of the virus in reptile species. An eastern mud turtle (*Kinosternon subrubrum*) captured on the SRS in April 2014 exhibited clinical signs of a ranaviral infection, including oral plaque and conjunctivitis. Quantitative PCR analyses of DNA from ocular, oral, nasal, and cloacal swabs were all positive for *Ranavirus*, and sequencing of the template confirmed infection with a FV3-like ranavirus. Histopathologic examination of postmortem tissue samples revealed ulceration of the oral and tracheal mucosa, intracytoplasmic epithelial inclusions in the oral mucosa and tongue sections, individualized and clusters of melanomacrophages in the liver, and bacterial rods located in the liver, kidney, heart, stomach, and small intestine. This is the first report of morbidity and mortality of a mud turtle with a systemic ranaviral infection.

Key Words: Amphibian; Chelonian; Frog virus 3; FV3; Iridovirus; Savannah River Site; Systemic infection; Zoonosis

INTRODUCTION

Amphibian and reptile populations are reported to be in the midst of a sixth mass extinction threatening global biodiversity (Daszak et al. 1999, Gibbons et al. 2000). Many factors are attributed to this massive decline, including emerging infectious diseases (Green et al. 2002, Collins & Storfer 2003, Stuart et al. 2003). While much research is focused on understanding the effects of ranaviruses on amphibian populations (Brunner et al. 2007, Haislip et al. 2012, Blaustein et al. 2012), few studies focus on reptilian populations (Sutherland et al. 2014). The genus *Ranavirus* (family *Iridoviridae*) has the ability to infect across fish, amphibians, and reptiles (Brenes et al. 2014). Amphibian mortality and morbidity events across five continents have been attributed to this pathogen (Speare & Smith 1992, Green et al. 2002, Fox et al. 2006, Ariel et al. 2009, Geng et al. 2011), but less is understood about its impacts on reptilian populations, particularly chelonians (Allender et al. 2006).

Eastern box turtles (*Terrapene carolina*) are among the most commonly reported turtle species associated with ranaviral die-off events (De Voe et al. 2004a, Allender et al. 2006, 2011, Currylow et al. 2014). However, surveillance and experimental challenge studies demonstrate that semi-aquatic turtles, such as red-eared sliders (*Trachemys scripta elegans*), can also become infected with ranaviruses (Allender et al. 2013, Goodman et al. 2013). The eastern mud turtle (*Kinosternon subrubrum*) is a lentic wetland, bottom-dwelling species that uses terrestrial ecosystems for dispersal and aestivation (Gibbons 1983, Buhlmann & Gibbons 2001). Rare, cryptic or secretive host species often have poorly understood life histories and small population sizes (Cecala et al. 2013). Eastern mud turtles reside in similar wetland habitats to species of amphibians

susceptible to ranaviruses in the Southeast, like pond-breeding salamander species, American bullfrogs (*Lithobates catesbeiana*; Hoverman et al. 2012) and green frogs (*Lithobates clamitans*; Gray et al. 2007), which are known carriers of ranaviruses. It is important to understand the potential impact of ranaviruses on the entire wetland community, including new host species or potential reservoirs for the virus.

METHODS

Background

The Savannah River Site (SRS) is an 80,000 ha Department of Energy installation located in west-central South Carolina, and first established in 1951. The SRS encompasses a variety of contaminated and reference (i.e., uncontaminated) wetland ecosystems including a network of reference wetlands known as Risher Pond sloughs. These heavily vegetated, temporary wetlands within the Savannah River floodplain are often inundated with water from nearby streams during years of heavy rainfall (Willson et al. 2005). Association with nearby streams creates the opportunity for amphibians, fish, crayfish, aquatic snakes, turtles, and a variety of other species to colonize and inhabit these wetlands.

On 12 April 2014 we opportunistically hand-captured an eastern mud turtle found resting along the water's edge of one of the Risher sloughs as a part of a herpetology class field trip. Mud turtles are common residents along with spotted turtles (*Clemmys guttata*) and common snapping turtles (*Chelydra serpentina*). Periodic aquatic trapping in the Risher Slough system (primarily between 2002-2007) has yielded >150 mud turtle captures but none with clinical symptoms of disease or infection. We kept the individual

in captivity for 17 days due to pronounced lethargy, observable oral plaque (Fig. 1), conjunctivitis and mucus discharge from both eyes (Fig. 2) and cloaca. On 25 April, we took oral, cloacal, ocular and nasal swabs and stored them in a -20 degree freezer. The individual died during the evening of 28 April or early the following morning, with the necropsy taking place on 29 April.

Molecular Methods

We extracted DNA from the swabs using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's instructions. We used a quantitative PCR TaqMan assay (Taqman® primers, FAM dye labeled, Applied Biosystems, Carslbad, CA) following the methods of Allender et al. (2013) and using an iCycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). We ran each sample four times on the same plate and included a serial dilution of *Ranavirus* standard from 10^6 to 10^1 viral copies per µL. The standard was frog virus 3 (FV3) isolate ID#061405 from leopard frog tissue. To confirm viral identity using sequencing, we then ran a second PCR with extracted DNA using primers 4 and 5 from Mao et al. (1997), purified the PCR products with Exonuclease I and Shrimp Alkaline Phosphatase enzymes (New England Biolabs, Ipswich, MA, USA) and bidirectionally sequenced them using BigDye v3.1 (Life Technologies, Carlsbad, CA, USA) and following manufacturers protocols. We ran the sequencing reactions on an ABI 3130xl (Life Technologies, Carlsbad, CA, USA) and assembled, aligned, and edited the sequences using the program Sequencher v5.2.4 (Gene Codes Corporation, Inc, Ann Arbor, MI, USA). No other pathogens were tested in this animal.

Histopathology

We performed a postmortem necropsy to collect samples included major viscera (liver, testes, kidneys, spleen, stomach, intestines, lung, and trachea), eyes, and brain. Following collection, we fixed samples in 10% buffered neutral formalin and submitted them to the University of Georgia's College of Veterinary Medicine Infectious Disease Laboratory, where they were dehydrated in graded alcohols and embedded in paraffin wax. Cassettes of paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E) stains.

RESULTS

Molecular

Based on qPCR results, all samples (oral, ocular, nasal, liver and cloacal) were definitively positive for FV3-like ranavirus. The viral loads per nanogram of extracted DNA were highest in the oral (82,828 ± 12,094) and ocular swabs (73,071 ± 5,893) and lowest in the cloacal swabs (5,389 ± 303). Our sequence analysis resulted in a 510bp sequence (Genbank Accession # KM114262) that, based on NCBI's Basic Local Alignment Search Tool, most closely matched the type *Ranavirus*, FV3. The 510bp fragment we sequenced corresponds to bases 56-565 of the FV3 complete major capsid protein coding sequence (Accession #FJ459783) and differs by three base pairs. The nucleotide differences result in two synonymous changes (G to C at position 150 of cds; C to T at position 354 of cds) and one non-synonymous change (C to A, Leu to Met, at position 400 of the cds).

Histopathology

Histologic examination revealed individualized and clusters of melanomacrophages, and multiple small colonies of bacterial rods in the sinusoids of the liver (Fig. 3). The renal tubules in the kidney were lined by degenerate epithelial cells with vaculoation and necrosis. Intravascular bacterial rods were also present in examined kidney, heart, vessels in the mesentery attached to the small intestine, and spleen sections. Sections of the trachea contained complete ulceration of the mucosa covered with intraluminal necrotic cellular debris and bacteria. The adjacent esophagus was deeply ulcerated and covered by caseous exudate. Oral mucosa and tongue sections had multifocal necrotic to ulcerated foci with hemorrhage, basal necrosis, and degeneration within the lamina propria with multifocal superficial bacterial basophilic inclusion bodies (Fig. 4). Several epithelial cells in and around these foci contained small round intracytoplasmic basophilic inclusion bodies. Within the conjunctiva, there were locally extensive areas of ulceration and necrosis covered with septic caseous exudate. There was also intraluminal caseous exudate and intravascular bacteria within the nasal cavity.

DISCUSSION

Ranavirues are a group of emerging pathogens affecting chelonian populations throughout the United States (Johnson et al. 2008). Ranaviruses, specifically FV3 or FV3-like viruses, have been associated with mortality events in species such as the leopard tortoise (*Geochelone pardalis pardalis*) (Benetka et al. 2007), eastern box turtle (De Voe et al. 2004), and soft-shelled turtle (*Trionyx sinesis*)(Huang et al. 2009, Chinchar

& Waltzek 2014) in captivity. To our knowledge, this is the first case of morbidity and mortality and subsequent detection of FV3-like ranavirus from a wild eastern mud turtle.

Clinical, histological, and molecular results were all consistent with a FV3-like ranavirus infection contributing to the morbidity and mortality in this mud turtle. The turtle displayed severe clinical signs and symptoms of a ranaviral infection upon capture, with a noted progression of infection while being held in captivity. Symptoms included pronounced weakness and lethargy in combination with clinical signs including swollen eyes, discharge from the nose and cloaca, and presence of white and yellow plaque on the palate and tongue. Histological analyses detected lesions in almost every tissue examined, with intracytoplasmic inclusions in the oral cavity and tongue epithelium that have been recorded in previous ranaviral cases (De Voe et al. 2004). Finally, the detection of FV3-like ranavirus from systemic lesions and liver samples also supports the diagnosis of a ranaviral infection as a contributing causal factor. The mud turtle may have been compromised due to a secondary bacterial infection, causing acute septicemia and increasing the risk of and contributing to the mortality of the animal. Further investigation by culturing would be necessary to determine the cause of sepsis recorded in this turtle and to identify the associated enterobacteria.

Risher sloughs, the location of capture, was tested for ranaviruses in amphibian populations during 2011-2012, resulting in a 27% prevalence of the disease in sampled amphibians (17/63 positive individuals; unpublished data). Amphibians have not been tested since 2012, thus the current presence and prevalence of ranaviruses in this amphibian population is unknown. Although the mud turtle in this case study is the first

turtle from the SRS tested for *Ranavirus*, we have not previously observed clinical signs in any turtle species in any wetland system on the SRS.

The results reported in this note expand the list of turtle species susceptible to ranaviruses and highlights the need to investigate cryptic and secretive species when studying disease dynamics. Species such as mud turtles may be exposed to ranaviral infections at an increased rate due to their associated life history strategies. Eastern mud turtles often live in lentic wetland habitats that serve as breeding habitat for amphibians. In addition, eastern mud turtles will scavenge on dead fish and other types of carrion, potentially increasing their exposure to ranaviral infections (Buhlmann et al. 2008). Future studies should survey a wide array of potential hosts to better inform conservation efforts.

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APPENDIX B

SURVEY OF AQUATIC TURTLES ON THE SAVANNAH RIVER SITE, SC FOR PRESENCE OF RANAVIRUS[4]

[4] Winzeler, M. E.. D. L. Haskins, S. L. Lance, and T. D. Tuberville. Submitted to *Journal of Wildlife Diseases* 12/15/2015

ABSTRACT

Ranaviruses have the ability to infect amphibians, fish, and reptiles, and have caused multiple amphibian die-off events in the United States and Europe. Prevalence of the virus in amphibian populations is much more commonly studied than in chelonian populations. We examined blood samples (N=288) during 2008-2014 from eight native aquatic turtle species on the Savannah River Site (SRS), South Carolina using banked blood samples from long-term mark-recapture efforts. We did not detect ranavirus in any individual, suggesting low presence or prevalence of ranavirus-like infections in aquatic turtles across the SRS during the years tested. Future studies should collect multiple sample types to test for this pathogen and should continue to monitor chelonians yearly and at the landscape-level.

Key Words: Chelonian; Ranavirus; South Carolina; Survey

Surveys of amphibian populations for infectious disease prevalence is common throughout much of the world (Carnaval et al., 2006; Keitzer et al., 2011; Kik et al., 2011; Reshetnikov et al., 2014), particularly after a die-off event is recorded (Green et al., 2002; Greer et al., 2005). However, ranaviruses not only infect amphibian populations but can also be found in wild and captive chelonian populations (Johnson et al., 2008; Allender et al., 2011). Transmission can occur between (Brenes et al., 2014), so it is necessary to survey all potential hosts to gain a full understanding of the disease presence and prevalence in an area.

The United States Department of Energy's Savannah River Site (SRS) near Aiken, SC, has a long history of herpetofauna research (Gibbons et al., 1997), but few studies have focused on the impacts of diseases on these populations (Daszak et al., 2005; Peterson et al., 2007). Recent studies on the SRS are focusing on ranaviruses in amphibian populations, while past studies focused on the prevalence of another amphibian pathogen, *Batrachochytrium dendrobatidis* (Daszak et al., 2005; Peterson et al., 2007), that does not infect reptiles. The presence of ranavirus-like infection in chelonian populations on the SRS was unknown until the mortality of a ranavirus-infected eastern mud turtle (*Kinosternon subrubrum*) was documented in 2014 (Winzeler et al., 2015). We subsequently tested blood samples collected from chelonians over a seven year period to better understand the presence and prevalence of ranaviruses on the SRS.

A total of 8 chelonian species were sampled (Table 1) across 23 locations on the SRS from 2008-2014. These sampling locations encompass many habitats such as Carolina bays, permanent wetlands and reservoirs, and coal ash settling basins. We

captured turtles using baited hoop traps and opportunistically by hand and collected blood samples that were frozen for future analysis.

Blood DNA extraction followed the protocols in the DNeasy Blood and Tissue Kit (Qiagen©, Valencia, CA). We determined DNA concentrations using a NanoDrop (Nanodrop Spectophotometer ND-1000, Thermo Scientific). After normalizing each individual sample to 20ng/μL of DNA, we combined 10μL samples of 5 individuals into a single well on a 96-well plate until all 288 samples were grouped. We pooled samples first by species of turtle and then within species by month and year the sample was taken. The plate of pooled samples was vortexed at least 30 seconds to ensure that DNA was mixed well.

We used quantitative PCR (qPCR) to test samples for presence of FV3-like infection. A reaction consisting of 2x TaqMan Platinum PCR Supermix-UDG with ROX, 1x TaqMan primer-probe (TaqMan® primers, FAM dye labeled, Life Technologies, Carlsbad, CA), 2.5 μL of pooled DNA, and water to a final volume of 12.5 μL. We followed the thermal profile developed in (Allender et al., 2013a) and ran our reactions on a BioRad C1000 Touch Thermal Cycler and CFX96® Real-Time System. We ran each pooled well and, if positive, individual samples from the pool, in triplicate, with a negative water control, and a serial dilution of a positive standard (10 to 10^6) cultured from a southern leopard frog (*Lithobates sphenocephalus*) on every plate. Each standard was replicated at least 3 times, with the 10 and 10^2 standards replicated 5 and 4 times respectively. A standard curve was generated for each plate, which allowed us to determine the number of viral copies in each sample. Samples with C_t values higher than the 10 standard were not recorded as positive.

A total of 288 blood samples were collected from 2008-2014 and tested for ranaviral infection with no evidence of active ranaviral infection. Several of the species we tested have exhibited ranaviral infection and mortality in prior surveys, experimental trials, or in captivity (Chen et al., 1999; Johnson et al., 2007; Allender et al., 2011; Goodman et al., 2013; Currylow et al., 2014). Studies have demonstrated that common ranaviral sampling methods (whole blood, oral swabs, cloacal swabs) for turtles can yield false-negative results (Goodman et al., 2013). A previous study found that 50% of their infected box turtles (*Terrapene carolina*) tested positive when using oral swabs but were whole blood negative (Allender et al., 2011). Conversely, Allender et al., (2013b) showed that oral and cloacal swabs can yield false-negative results when compared to post-mortem tissue and whole blood samples in red-eared sliders (*Trachemys scripta elegans*). It could be that, like amphibians, there are species-specific differences (i.e., viral shedding, immune responses) in chelonians that will determine which sampling method should be employed (Schock et al., 2008).

Chelonians can act as reservoirs for FV3-like ranavirus, and transmission can occur between taxa (Brenes et al., 2014). Ranaviruses can persist in aquatic environments for long periods of time (Harp and Petranka, 2006; Brunner et al., 2007). Nazir et al., (2012) noted that FV3-like ranaviruses can persist in pond water for 3-4 weeks at 20°C and 5-6 weeks at 4°C. In spring of 2014 an eastern mud turtle (*Kinosternon subrubrum*) from the SRS died with FV3-like ranaviral infection (Winzeler et al., 2015), indicating that the pathogen is present in chelonian populations. A previous study also shows that 37.4% of amphibians tested in 2011-2012 were positive for ranaviral infection (authors' unpublished data) at many of the same locations used for this study. Due to the various

routes that ranaviruses can infect their hosts (i.e., direct contact, water exposure, and possibly consumption of prey; Johnson et al., 2007; Brunner et al., 2007), it is imperative that semi-aquatic and aquatic chelonian species are included in prevalence surveys and transmission research.

Further research is needed to elucidate species-specific trends of this virus in chelonians. Some species may serve as pathogen reservoirs, maintaining the virus in the aquatic system even if they are not themselves exhibiting signs of disease (Johnson et al., 2012). In addition, infected individuals may exhibit altered behavior (e.g., lethargy) and be unlikely to be encountered using traditional trapping techniques. Even though the dynamics of ranavirus in chelonians are currently poorly understood, it is critical to continue monitoring multiple species and banking multiple sample types (i.e. swabs and blood) to detect virus outbreaks, which in other ectotherms can be short in duration yet result in high rates of mortality.

We thank the many researchers who dedicated their time and skills in capturing and collecting samples from turtles on the SRS since 2008, especially Bess Harris, Matt Hamilton, Brian Metts and Kurt Buhlmann. We also would like to thank Caitlin Rumrill and Imogene Davis for assistance with lab work. This study complied with guidelines set by the University of Georgia Animal Care and Use Committee under A2008 11-035-Y2-A0 and A2013 12-008-Y2-A0. This research was partially supported by the U. S. Department of Energy under Award Numbers DE-- FC09-□07SR22506 to the University of Georgia Research Foundation, and was also made possible by the status of the SRS as a National Environmental Research Park (NERP).

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Table

Table 1. Total number of sampling locations and individuals for which blood samples were collected from eight species of aquatic turtles on the Savannah River Site, SC during 2008-2014 (* Total number of unique locations sampled across species)

Scientific name	Common name	N	No. of Locations
Trachemys scripta	Yellow-bellied slider	168	14
Kinosternon subrubrum	Eastern mud turtle	61	5
Terrapene carolina	Eastern box turtle	23	17
Sternotherus odoratus	Common musk turtle	13	7
Chelydra serpentina	Common snapping turtle	13	6
Clemmys guttata	Spotted turtle	6	3
Apalone spinifera	Spiny softshell turtle	1	1
Pseudemys floridana	Florida cooter	1	1
Totals	8	288	23*