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Mechanisms Contributing to the Emergence of Ranavirus in Ectothermic Vertebrate Communities

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I am submitting herewith a dissertation written by Roberto M. Brenes entitled "Mechanisms Contributing to the Emergence of Ranavirus in Ectothermic Vertebrate Communities." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Natural Resources.

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Mechanisms Contributing to the Emergence of Ranavirus in Ectothermic Vertebrate Communities

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Dedication

To Mabeluca, Claudito, and Kika; my past, present, and future

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ABSTRACT

Ranaviruses have been linked to amphibian die-off events in ectothermic vertebrates worldwide. Differences in susceptibility and capacity of transmission among and within classes are poorly understood. My goal was to determine possible mechanisms influencing susceptibility to ranavirus infection in amphibian species and other aquatic vertebrate taxa, as well as the capacity of transmission between classes and the effects of amphibian community composition on ranavirus transmission. I tested 16 amphibian species from USA, Europe, and the pet trade, expanding an existing database developed by the Center for Wildlife Health to 35 amphibian species from 9 families. I also tested the susceptibility of 5 fish and 3 turtle species by exposure to a panel of ranaviruses from amphibian, fish and reptilian hosts under laboratory conditions. I used outdoor aquatic mesocosms to explore if certain species functioned as amplification hosts in a semi-natural environment. All vertebrate classes tested (amphibian, reptile, and fish) presented variability in susceptibility. Amphibians were most susceptible to ranavirus, but no phylogenetic relationship with susceptibility was detected. Susceptibility was related to life history characteristics of amphibian hosts. Fast-developing species that bred in temporary wetlands during spring showed higher susceptibility to ranavirus. Further, for one of the isolates, pathogenicity increased as distance between host population and isolate location increased. Fish and turtle species showed low susceptibility to ranavirus, but could function as reservoirs for ranavirus due to documentation of subclinical infections. Transmission experiments demonstrated that ranavirus could be transmitted between classes, with greatest mortality when infected turtles or fish transmitted the virus to amphibians. Finally, I showed that community composition affects ranavirus transmission and mortality in larval amphibians. Wood frog larvae functioned as amplification hosts to spotted salamander (*Ambystoma maculatum*) and caused an outbreak in chorus frog (*Pseudacris feriarum*). My results demonstrate that ranaviruses can infect multiple hosts from different classes with different susceptibilities, contributing to its persistence in the environment and recurrent outbreaks. My results can be used to identify potential species of high risk to ranaviral disease and highlight the need to understand host community to predict ranavirus outbreaks and develop conservation strategies to mitigate emergence of ranaviral disease.

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CHAPTER I

INTRODUCTION

According to the millennium ecosystem assessment, current extinction rates around the world are estimated to be 100 – 1000 times higher than in the past, and if this trend continues; future extinction is expected to be 10 –100 times higher than current rates (Mace et al. 2005, Dawson et al. 2011). Some authors have suggested that based on the size and velocity of the current local and global extinctions, these large scale biodiversity declines could be considered part of the beginning of the sixth mass extinction which will have detrimental impacts on global biodiversity and the functioning of a myriad of ecosystem processes (Pereira et al. 2010, Barnosky et al. 2011, Whiles et al. 2013). These declines in biodiversity are widespread and affect all taxonomic groups. Estimates from the IUCN red list indicate that 12% of birds, 23% of mammals, 31% of gymnosperms, 33% of corals, and 32% of amphibians are under threat of extinction (IUCN 2011). While most global ecosystems are affected by these population declines, freshwater aquatic environments are considered the most imperiled habitats, suffering from biodiversity declines and loss of ecosystem function (Colon-Gaud et al. 2010, Connelly et al. 2011, Whiles et al. 2013). The extensive loss in biodiversity in freshwater ecosystems can be observed in the current global declines of all its inhabitants, including amphibians (Alexander and Eischeid 2001, Alford et al. 2001, Stuart et al. 2004, Alton et al. 2009, Hayes et al. 2010, Barreiro and Tung 2012, Blaustein et al. 2012), fish (Barbour et al. 1999, Wright and Flecker 2004, Januchowski-Hartley et al. 2011, Daw et al. 2012), and reptiles (Gibbons et al. 2000, Brodman et al. 2005, Schlaepfer et al. 2005). Freshwater turtles have been identified as the most threatened vertebrate group in the world (Chessman 2011, Christiansen et al. 2012).

Although biodiversity declines have been related to factors such as habitat fragmentation (Greer and Collins 2008, Bergerot et al. 2010), overexploitation (O'Brien et al. 2003, Primack 2010), invasive species (Doody et al. 2009, Johnson et al. 2011), and climatic changes (Pounds et al. 2006, Pounds et al. 2007, Lips et al. 2008, Lowe 2012), emerging infectious diseases have been identified as the most important factor responsible for population declines of freshwater organisms (Carey 2000, Daszak et al. 2003, Mendelson et al. 2006, Hayes et al. 2010). The effect of emerging infectious diseases in population declines is most evident in amphibian populations, and constitutes a growing threat to global amphibian biodiversity (Daszak et al. 2003, Daszak et al. 2005, Collins 2010, Keesing et al. 2010, Kiesecker 2011). Two major emerging infectious diseases have been associated with mass mortality of amphibians across the globe (Halliday 2007, Gray et al. 2009, Alford 2010, Miller and Gray 2010, Muths and Hero 2010, Miller et al. 2011a); chytridiomycosis and ranaviral diseases (Kiesecker et al. 2004, Hayes et al. 2010). While *Batrachochytrium dendrobatidis* (Bd) has been identified as the cause of several amphibian population declines in tropical areas (Lips et al. 2006, Whitfield et al. 2008, Rovito et al. 2009, Voyles et al. 2009, Kilpatrick et al. 2010), ranaviruses have been responsible for the majority of pathogen-associated die-off events in temperate regions like North America and Europe (Greer et al. 2005, Pearman and Garner 2005, Kik et al. 2011, Miller et al. 2011a). Whereas Bd-associated declines are generally widespread across a geographic landscape (Carnaval et al. 2006, Puschendorf et al. 2006, Murray et al. 2010, Olson et al. 2013, Richards-Hrdlicka et al. 2013), ranavirus-associated mortality events are often localized and may affect a single pond or single population (Duffus et al. 2008, Teacher et al. 2010, Torrence et al. 2010).

Ranaviruses are members of the family Iridoviridae which encompasses five genera of large dsDNA viruses whose virions display icosahedral symmetry and are usually 120 – 200 nm

in diameter (Chinchar et al. 2009a, Chinchar et al. 2011b, Lesbarreres et al. 2012). Iridoviruses can infect vertebrate and invertebrate hosts. Currently, five genera of iridoviruses are recognized (King et al. 2012); two genera known mostly to infect arthropods (Iridovirus and Chloriridovirus), and three genera (Lymphocystivirus, Megalocytivirus, and Ranavirus) that infect lower ectothermic vertebrates. In addition to their host preferences (i.e., vertebrates or invertebrates), ranaviruses, megalocytiviruses, and lymphocystivirus differ from the other two genera by their high level (about 25%) of cytosine methylation (Mao et al. 1999b, Jancovich et al. 2003b). Aside from the differences in virion and genome sizes, members of the Iridoviridae family are generally similar, particularly on the major capsid protein (MCP), which shows marked sequence conservation and could explain the high diversity of suitable hosts (Chinchar and Mao 2000, Chinchar et al. 2011b, Robert and Chinchar 2012).

Currently, the International Committee on Taxonomy of Viruses recognizes six species of ranaviruses known to infect lower vertebrate hosts (King et al. 2012), three of the species; epizootic hematopoietic necrosis virus (EHNV), European catfish virus (ECV), and Santee-cooper ranavirus (SCRV), are exclusively found in fish hosts (Bigarre et al. 2008, Chinchar et al. 2009b, Whittington et al. 2010, Jensen et al. 2011b, Vesely et al. 2011), while the other species are known to infect fish, reptiles and amphibian hosts. For example, the ranavirus Ambystoma tigrinum virus (ATV) has been shown to infect amphibian (Jancovich et al. 2003a, Collins et al. 2004) and fish host (Picco et al. 2010). Similarly, the Bohle iridovirus (BIV) can infect fish (Moody and Owens 1994a) and amphibians (Speare and Smith 1992). The type species of ranavirus is the Frog Virus 3 (FV3) and has been documented infecting fish (Moody and Owens 1994a, Whittington et al. 1996, Mao et al. 1999a, Chinchar et al. 2001, Gobbo et al. 2010, Whittington et al. 2010, Jensen et al. 2011a), terrestrial turtles (Marschang et al. 1999, De Voe et

al. 2004, Johnson et al. 2007, Johnson et al. 2008, Johnson et al. 2010, Allender et al. 2011), semi-aquatic turtles (Johnson et al. 2006, Johnson et al. 2007), fully aquatic turtles (Chen et al. 1999), snakes (Hyatt et al. 2002), lizards (Marschang et al. 2005, Alves et al. 2008, Marschang 2011), and anurans (Greer et al. 2005, Mazzoni et al. 2009, Gahl and Calhoun 2010, Geng et al. 2011, Hoverman et al. 2011b, Miller et al. 2011a).

Amphibian susceptibility to ranaviruses has been reported to be species specific, affecting some species more aggressively than others, with mortality ranging from 0 to 100% for different species when tested under laboratory conditions (Schock et al. 2009, Hoverman et al. 2011b). These differences in susceptibility are also seen among developmental stages (Brunner et al. 2004, Hoverman et al. 2010, Haislip et al. 2011). For example, wood frog (*Lithobates sylvaticus*) larvae experienced 100% mortality, whereas northern leopard frogs (L. pipiens) and American bullfrogs (*L. catesbeianus*) experienced less than 40% and 10% mortality after exposure to a FV3-like ranavirus, respectively (Hoverman et al. 2011). Differences in susceptibility observed among species have been suggested to be influenced by different biotic or abiotic factors shaping the natural history of the species, including phylogeny, evolutionary history, habitat stressors, immunocompetence capacity, and molecular structure of the ranavirus (Chinchar et al. 2009b, Teacher et al. 2009, Haislip et al. 2011, Hoverman et al. 2011b, Grayfer et al. 2012).

Other factors influencing species susceptibility to ranavirus are the routes of transmission and possibly the structure of the aquatic community. Ranavirus transmission is highly efficient and can occur through direct contact among individuals (Brunner et al. 2004, Hoverman et al. 2010), or indirectly from infected to susceptible hosts via environmental transmission (Brunner et al. 2007, Breban 2013). According to Paull et al. (2012), the capacity of a host to transmit a pathogen is dependent on three main factors; the degree of contact that the host has with other

individuals, the degree of susceptibility of the host to the pathogen, and the amount of pathogen shed by the host. These characteristics will determine the contribution of a host to the pathogen in the environment and possibly transmission dynamics within a community (Lloyd-Smith et al. 2005, Paull et al. 2012). Hence, the propagation of ranavirus in a determinate environment will be a direct response of the susceptibility of each species inhabiting that environment, their capacity to transmit the pathogen, and the inter- and intra-specific interactions among all members of the community (Woolhouse 2002, Lloyd-Smith et al. 2005, Paull et al. 2012).

The objective of my dissertation was to identify factors contributing to the emergence of ranaviral disease in amphibians at the organismal, community, and ecosystem levels. To determine the effects of ranaviral diseases at the organismal level, I tested the susceptibility of 16 amphibians, 5 fish and 3 turtle species to ranavirus and for the amphibian species related susceptibility to host characteristics. To determine possible community effects of ranavirus, I tested the capacity of ranavirus to transmit among fish, reptiles, and amphibians in controlled laboratory experiments. I also tested the effects of larval amphibian community composition on ranavirus transmission dynamics in outdoor aquatic mesocosms.

My dissertation is written in manuscript style and divided into four chapters corresponding to different studies. In chapter II, I focus on determining the susceptibility among 16 amphibian species. I combined my data with Hoverman et al. (2011), and ran analyses on 35 species from 9 families to identify if there was a phylogenetic signal for host susceptibility (i.e., infection prevalence) and whether life-history factors could be used to identify high risk species. Species that I analyzed included 22 from the southeastern USA, six from the Midwestern USA, one from the southwestern USA, three from the northwestern USA, one species from the UK, and two species from the pet trade. Based on the results of Hoverman et al. (2011) and other

studies, I hypothesized that: 1) Susceptibility among species would be highly variable, and 2) that the variability in susceptibility observed among species would be the result of evolutionary history among individuals and species-specific natural history traits.

In chapter III, I focused on determining the susceptibility of five fish and three turtle species to three different FV3-like ranaviruses isolated from different vertebrate ectothermic hosts (amphibian, reptile, and fish). The species I tested included: tilapia (*Oreochromis niloticus niloticus*, Cichlidae), channel catfish (*Ictalurus punctatus*, Ictaluridae), mosquito fish (*Gambusia affinis*, Poecilidae), bluegill (*Lepomis macrochirus*, Centrarchidae), and fathead minnow (*Pimephales promelas*, Cyprinidae), Florida soft-shell turtle (*Apalone ferox*, Trionychidae), eastern river cooter (*Pseudemys concinna*), and Mississippi map turtle (*Graptemys kohni*, Emydae). I hypothesized that, similar to amphibian species, susceptibility among fish and turtle species would be highly variable.

In chapter IV, I focused on determining if syntopic species from different ectothermic classes known to be susceptible to ranaviruses, specifically the red-eared slider turtle (Trachemys scripta), the mosquito fish (G. affinis), and the Cope's gray treefrog (*Hyla chryscocelis*), were able to transmit a FV3-like ranavirus to naïve individuals under laboratory conditions. Based on previous observations and studies, I hypothesized that transmission between classes would occur but infection prevalence would be variable among species and related to host susceptibility.

In chapter V, I focused on determining if the outcome of a ranaviral disease outbreak in an amphibian community in semi-natural conditions would depend on which species was initially exposed to the virus. Specifically, I explored whether certain species exposed to ranavirus could amplify mortality rates or initiate ranavirus outbreaks in syntopic species. I

hypothesized that the occurrence of species and community-level outbreaks would depend on the level of susceptibility of the species first exposed to the pathogen, where highly susceptible species would initiate outbreaks more often than less susceptible species.

CHAPTER II

LIFE HISTORY MATTERS: HOST CHARACTERISTICS AFFECT SUSCEPTIBILITY TO AN EMERGING PATHOGEN

Ranaviruses have been linked to amphibian die-off events across the globe, with susceptibility reported as being highly variable among species and age classes. Initial experiments suggest that susceptibility may be related to species natural history and ancestral phylogenetic lineages. In 2011 and 2012, I tested the susceptibility (infection prevalence) of 16 amphibian species via standard water bath exposures to three FV3-like ranavirus isolates, and combined these data with 19 amphibian species previously tested by Hoverman et al. (2011). Phylogenetic comparative methods demonstrated high variability in susceptibility among 35 amphibian species with no significant phylogenetic signal. Life history characteristics that were positively correlated with susceptibility included use of temporary wetlands during breeding, fast-developing larvae, and spring breeders. Highly susceptible species included uncommon species like the gopher frog (e.g., *Lithobates capito*) and common species like the wood frog (*L. sylvaticus*). My results can be used to identify possible species at greatest risk to ranavirus, and could be combined with natural population demographics to predict population outcomes following a ranavirus outbreak.

INTRODUCTION

Ranaviruses are a diverse group of pathogens known to infect multiple amphibian species (Duffus et al. 2008, Schock et al. 2008, Schock et al. 2009, Hoverman et al. 2010, Hoverman et al. 2011b). Ranaviruses are known to infect at least 72 amphibian species in 14 families around the world (Miller et al. 2011b). Susceptibility to ranaviruses among amphibian species has been described to be highly variable, changing greatly among amphibian species and ranavirus isolates. For example, Schock et al. (2008) reported that Ambystoma tigrinum virus (ATV) caused 100% mortality to tiger salamander (Ambystoma tigrinum) larvae, but Frog Virus 3 (FV3) only resulted in 20% mortality. In other experiments, FV3 has been shown to be very pathogenic to a variety of species (e.g., Haislip et al. 2011, Hoverman et al. 2011b). For example, the pathogenicity of FV3-like ranaviruses is very high for wood frog larvae (Harp and Petranka 2006, Hoverman et al. 2010, Warne et al. 2010, Hoverman et al. 2011b, Warne et al. 2011), whereas other amphibian species such as the American toad (*Anaxyrus americanus*) or the eastern narrow-mouthed toad (*Gastrophryne carolinensis*) exhibited low to no susceptibility to ranavirus in laboratory challenges (Hoverman et al. 2011).

To determine differences in susceptibility among amphibian species from eastern North America, Hoverman et al. (2011) performed standard water-bath challenges with two ranavirus isolates for 19 species. Infection prevalence among species ranged from 0 to 100%, and was dependent of the type of ranavirus isolate. Hoverman et al. (2011) related differences in degrees of susceptibility among species to the phylogenetic and natural history traits of the species. The authors grouped species based on phylogenetic relationships (family, genera), and other traits such as length of time in larval stage (hydroperiod), breeding habitats and breeding time of adult, body size at metamorphosis, duration of the egg stage, adult body size, time to maturity, and

species range size. The authors reported that Ambystomatidae, Hylidae, and Ranidae tended to have low, intermediate, and high susceptibility to ranaviral infection, respectively. The authors also related high ranavirus susceptibility to three main natural history traits: tendencies to breed in temporal habitats, narrow geographic distributions, and fast developmental time. The authors hypothesized that species that breed in temporal habitats may have experienced limited opportunities for co-evolution with ranavirus (Anderson and Graham 1967, May and Anderson 1979, Connell 1980), perhaps resulting in reduced immune system response. They suggested that fast developing larvae may be incapable of mounting a strong immune response as a consequence of energy re-allocation during development (Pfennig and Murphy 2000, 2002, Olalla-Tárraga and Rodríguez 2007, Warne et al. 2010, 2011). Species with limited geographic distribution may be subjected to greater occurrences of population isolation, which can reduce immune response to ranaviruses through genetic drift (Pearman and Garner 2005).

The natural history factors affecting the susceptibility of amphibian species described by Hoverman et al., (2011) represent an important step in understanding mechanisms influencing species susceptibility to ranaviruses. However, the database created by Hoverman et al. (2011) was restricted to species from eastern North America and only the well represented families (i.e., Ranidae, Hylidae, and Ambystomatidae) were included in the phylogenetic analyses. My objective was to test the susceptibility of 16 additional species and perform combined analyses on 35 species from 9 families following the methods of Hoverman et al. (2011). Further, I focused on attempting to secure additional species outside of eastern North America to increase robustness of my results. My goal was to determine if phylogenetic and life history trends documented by Hoverman et al. (2011) continued to hold true, and identify host characteristics that are consistent with high-risk infection to ranavirus.

METHODS

I added 16 amphibian new species from eight families to the 19 species previously tested by Hoverman et al. (2011, Table 2.1). In total nine amphibian families were tested: Ranidae (10 Spp), Hylidae (6 Spp), Bufonidae (3 Spp), Microhylidae (1 Spp), Scaphiopodidae (2 spp), Pipidae (1 sp), Ambystomatidae (8 Spp), Salamandridae (3 Spp), and Plethodontidae (1 Sp). All species (except pet trade species) were collected as egg masses from natural populations between 2011 and 2012 (Table 2.2). Pet trade species (*Cynops pyrrhogaster*, and *Xenopus laevis*) were collected opportunistically from local breeders. Species that were collected from colleagues were shipped overnight to the University of Tennessee (UT). All egg masses were hatched indoors at the UT Joe Johnson Animal Research and Teaching Unit (JARTU) in 11.7 L tubs with 4 L of aged water. Once hatched, larvae were moved outdoors to 324-L wading pools covered with 70% shade cloth lids that allow the larvae to experience natural diel temperature fluctuations and photoperiods. Individuals were kept in the wading pools until reaching Gosner stage 30 for tadpoles (Gosner 1960) and one month of age for salamander larvae. Developmental stage was standardized because susceptibility to ranavirus differs among developmental stages (Haislip et al. 2011). During captive rearing, anuran larvae were fed high protein fish food (TetraMin®, Blacksburg, VA), and salamander larvae were fed zooplankton (predominately Daphnia sp.) ad libitum. Zooplankton was raised in 1000-L outdoor cattle tanks as described by Hoverman et al. (2011). Water was changed every week to maintain high water quality. Each species was cultured separately and densities were maintained at <1 ind/L (Relyea 2002, Hoverman et al. 2011b).

Once the larvae reached the appropriate developmental stage, individuals were transported to the laboratory, housed individually in 2-L containers filled with 1-L of de-

chlorinated aged tap water and placed on 4 x 8-ft shelving units. Prior to the beginning of each experiment, five randomly assigned individuals were euthanized and tested for ranavirus using PCR; all pre-experimental individuals tested were negative for ranaviruses. All the experiments were conducted at 25°C on a 12:12 day:night photoperiod (Relyea and Werner 1999). I used 25°C as the standard temperature, because this is the average water temperature during summer in pond systems in mid-latitudinal United States (Schmutzer et al. 2008). Although variation exists in water temperature during larval development among amphibian species (according to breeding phenology), all species used in my experiments have distributions where their larvae can be exposed to 25°C during development. Standardizing at 25°C also removes potential confounding effects of temperature on immune function, host susceptibility and viral replication (Gray et al. 2007).

Virus isolates

During my experiments, I used three different FV3-like ranavirus isolates. Two of the isolates, the FV3 type species (Granoff et al. 1965) and a FV3-like isolate obtained from an American bullfrog that died during a mortality event inside a commercial ranaculture facility in Southern Georgia (Miller et al. 2007), were the same isolates used by Hoverman et al. (2011). Hoverman et al. (2011) found that the ranaculture isolate was more pathogenic than FV3. I added a third isolate obtained in 2009 from a dead marble salamander (*Ambystoma opacum*) larva collected during a die-off in Gourley pond at the Smoky Mountains National Park (Todd-Thompson et al. 2009).Virus was cultured and tittered by Dr. Rebecca Wilkes of the UT College of Veterinary Medicine. Virus was stored in a -80^{o}C freezer until used, thawed only once for experimentation, and used for only one species.

Trials

Each experimental trial was arranged in a randomized block design with four treatments and 20 replicate larvae per treatment, totaling 80 experimental units. Treatments included three ranavirus isolates (FV3, Great Smoky Mountains [SM], and Ranaculture [RC]) and the control. Eighty larvae (previously added to 2L containers) were randomly assigned to a viral treatment or a control treatment. Individuals assigned to a viral treatment received 10^3 PFU/mL of the appropriate virus isolate added directly to the container. This dosage is sufficient to induce ranaviral disease in amphibians and is environmentally relevant (Tweedell and Granoff 1968, Pearman et al. 2004, Rojas et al. 2005, Morales and Robert 2007, Hoverman et al. 2010). Given that larvae were ranavirus negative at the beginning of each experiment [as verified by PCR testing, Picco et al. (2007)], inoculations represented first-time exposure to the pathogen, which is standard in ranavirus-challenge experiments (Brunner et al. 2004, Hoverman et al. 2010). Control larvae were exposed to the same quantity of virus-free media (i.e., minimum essential media, MEM Eagle). Water bath exposures lasted three days, which is sufficient duration to initiate infection in anuran and salamander larvae (Hoverman et al. 2010, Haislip et al. 2011). During the 3-day virus exposure, no food was given to avoid unknown effects of food particles on transmission. After the inoculation period and every three days thereafter, water was changed (100% of volume) to maintain water quality. Amphibian larvae were fed ground fish food (TetraMin®) at a ratio of 12% of their body mass every three days (Relyea 2002, Hoverman et al. 2010). To determine the amount of food required, a separate sample of five non-experimental tadpoles treated identically to the controls was measured every feeding day to determine food ration amounts. Using non-experimental tadpoles reduced the likelihood of cross contamination among experimental units and eliminated introducing potential stress into the experiment

associated with weighing individuals. Salamander larvae were fed 3 mL of concentrated zooplankton daily (Hoverman et al. 2010, 2011).

Larvae were monitored twice daily for survival and morbidity (i.e., petechial hemorrhages, edema, and loss of equilibrium; Miller et al. 2011). Dead individuals and larvae that exhibited extreme morbidity consistent with ranavirus infection were removed from their containers, humanely euthanized (if still alive), necropsied, and sections of the liver and kidney collected for virus testing by qPCR analysis. The duration for all trials was three weeks (21 calendar days), which is sufficient duration for morbidity to be observed from ranavirus infection (Brunner et al. 2004, Brunner et al. 2005, Hoverman et al. 2010). At the end of each experiment, all surviving larvae were humanely euthanized by immersion in a solution of benzocaine hydrochloride diluted in 90% ethanol at a 5 ml/L dosage until cessation of ventilating. All procedures followed approved IACUC #2009 for the University of Tennessee.

To test for the presence of the virus, I ran quantitative PCR on all individuals. Genomic DNA (gDNA) was extracted from homogenates from liver and kidney samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). I used a QubitTM fluorometer and the Quant-iTTM dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA). Real-time PCR (qPCR) was used to verify the presence or absence of ranavirus DNA. The qPCR procedure followed that of Picco et al. (2007) and Hoverman et al. (2010). All samples were run in duplicate and an individual was declared infected if the qPCR cycle threshold (CT) was <30 for both samples. The CT was determined for our PCR system (ABI 7900Fast Real-Time PCR System; Life Technologies Corporation, Carlsbad, CA) by developing a standard curve using known quantities of virus. Four PCR controls were used for reference: positive control, negative control, FV3 control, and water.

Data Analysis: Isolate Effect

For the FV3 and RC isolates, all analyses were conducted using all 35 species tested. For the SM isolate, the 16 species tested between 2011 and 2012 were used. I used Mann-Whitney rank sum test to test for differences in susceptibility between the FV3 and RC isolates for the 35 species. A Kruskal-Wallis test was used to test for differences in susceptibility among FV3, RC and SM isolates for the 16 species tested in 2011 and 2012. Similar to Hoverman et al. (2011), I used the number of individuals that were infected with ranavirus and died as the index of host susceptibility. Total mortality after 21 days was not used, because some individuals died without detectable infections. Because I did not detect infection in any control individuals (exposed to free virus growth media), I excluded these individuals from the infection analyses. I used Person's correlation coefficient to quantify the linear relationship between infected prevalence and total mortality after 21 days. All analysis were performed using SAS 9.3 (SAS 2012) at $\alpha = 0.05$.

Data Analyses: Phylogeny

Because of the limited replication of species within families for the SM isolate, phylogenetic analyses were performed for the FV3 and RC isolates only. Following Hoverman et al. (2011), I used infection prevalence as the index of host susceptibility. I obtained phylogenetic information including the relationships among the species (i.e., tree topology) and the amount of change that has occurred along the tree (i.e., branch lengths), from previously published phylogenetic relationships among the species (Case 1978, Hillis et al. 1983, Shaffer et al. 1991, Pauly et al. 2004, Lemmon et al. 2007, Henrici 2009, Wiens et al. 2010, Newman and Rissler 2011, Pyron 2011). Branch lengths (i.e., divergence times) for the phylogeny were

obtained using TimeTree (Hedges et al. 2006) or published studies (Case 1978, Hillis et al. 1983, Shaffer et al. 1991, Pauly et al. 2004, Lemmon et al. 2007, Henrici 2009, Wiens et al. 2010; Figure 1). When divergence times were not available for a group, I divided the branch length equally among the taxa. I tested for a phylogenetic signal ($\alpha = 0.05$) in host susceptibility using the ‘‘Kcalc’’ function in the ‘‘picante’’ package of R statistical software (Blomberg et al. 2003, R Development Core Team 2008).

Data Analyses: Host species characteristics

I collected data from the literature on 13 species-level characteristics to explore their potential association with ranavirus susceptibility using phylogenetic comparative methods (Altig 1970, Conant and Collins 1998, Petranka 1998, McDiarmid and Altig 1999, Savage 2002, Altig and McDiarmid 2007, Wells 2007). The characteristics followed Hoverman et al. (2011) and included: hydroperiod of the breeding habitat, seasonal breeding time, duration of egg stage, duration of larval stage, size at metamorphosis, clutch size, adult body size, time to maturity, species range size, relationship to water (aquatic index), individual size at time of reproduction, egg size, aggregation behavior (gregarious or solitary; table 2.3). Breeding habitat hydroperiod was coded from 1 to 4 with 1 = ephemeral ponds that dry within weeks after filling, 2 = temporary ponds that dry in the early summer, 3 = semi-permanent ponds that dry in certain years, and 4 = permanent water bodies. If a species was reported as breeding in multiple habitat types with different hydroperiods, I recorded the mean hydroperiod duration of the reports as the response for the species. Breeding time was coded 1 = winter breeding, 2 = early spring, 3 = late spring, and 4 = summer. Species range size was estimated from available distribution maps. Aquatic index was coded from 1 to 3 depending on the dependence of the species to water: 1= only found in near water during reproduction, 2= usually found in or around water, 3= fully

aquatic. Egg size was coded 1 to 3 with 1= small eggs, 2= intermediate size eggs, and 3= large eggs. Aggregation behavior was coded 0= solitary (non-chorus) and 1= gregarious. In cases when was the substantial variation in information found for a characteristic or the information was presented as a range, I followed Hoverman et al. (2011) and calculated the mean of the reported range(s) as the response.

I identified host characteristics that were related to infection prevalence by developing all-possible models and using Akaike's Information Criterion (AIC) as a measure of performance (Anderson 2008). Models were sorted from lowest to highest AIC, and candidate models identified as those with AIC values within 2 units of the lowest AIC (i.e., $\Delta AIC < 2$). Model weights, variance (root mean-square error, RMSE), and adjusted coefficients of determination were presented as additional measures of model performance. For FV3 and RC isolates, model performance was poor so I performed model averaging across all-possible models to identify the overall weight of variable in the global model, model average standardized beta value (MA STβ), and the model average standard error (MA-SE) to identify the characteristics that were most important (Anderson 2008). Standardized beta estimates were presented with weights of host characteristics for an indication of strength and direction of association with host susceptibility. Analyses were performed using SAS® JMP 10 (SAS_Institute 2007).

RESULTS

Exposure to FV3 and RC isolates resulted in detectable infection after 21 days in 26 and 32 species, respectively, out of 35 species tested (Figure 2.2B, C). Infection prevalence was approximately 20% greater for the ranaculture isolate compared to the FV3 isolate ($U = 404.5$, $P = 0.014$; Figure 2.4). Infection prevalence was on average 72% greater for the RC isolate

compared to the FV3 isolate for six species (Figure 2.2.): common frog (*Rana temporaria*, 100% vs. 35%), green frog (*L. clamitans*, 95% vs. 5%), pickerel frog (*L. palustris*, 95% vs. 20%), Jefferson salamander (*Ambystoma jeffersonianum*, 95% vs.20%), and tiger salamander (*Ambystoma tigrinum*, 90% vs. 35%). Infection was detected for 14 of 16 species exposed to the SM isolate (Figure 2.3). Infection prevalence did not differ among FV3, RC and SM isolates (H=1.286, 2 df, P>0.23; Figure 2.5). However, for 3 species, susceptibility was on average 28% greater for the SM isolate (Figure 2.3): Northwestern salamander (*A. gracile*, 73% vs. 54%), yellow legged frog (*R. boylii*, 20% vs. 0%), and southern toad (*Anaxyrus terrestris*, 20% vs. 5%).

There was a strong correlation between mortality and infection prevalence for the FV3 (R^2 = 0.864, P = 0.024), RC (R^2= 0.926, P = 0.003), and SM (R^2=0.883, P = 0.011) isolates. For FV3, 10 species showed high susceptibility (>75% mortality), 15 species showed medium susceptibility (25% to 75%), and 10 species showed low susceptibility (<25%). For the RC isolate, the common frog (*R. temporaria*), Couch's spadefoot (*Scaphiopus couchi*), Southern leopard frog (*L. sphenocephala*), and ornate chorus frog (*Pseudacris ornata*) experienced 100% mortality when exposed to ranavirus (Figure 2.2 B). Eight additional species showed high susceptibility (75 – 95%) to the RC isolate, while 12 and 8 species showed moderate (30% to 60%) and low (5% to 25%) susceptibility, respectively (Figure 2.2 C). For the SM isolate, the ornate chorus frog and Couch's spadefoot experienced 100% mortality, while two species showed high susceptibility (87% to 90%), 5 species showed moderate (34% to 60%), and 5 species low susceptibility to the isolate (5% to 20%; figure 2.3). Control mortality was observed for several species (e.g., upland chorus frog, P. feriarum; four toed salamanders, *Hemidactylium scutatum*; African clawed frog, *Xenopus laevis*), but none of the control deaths were positive for ranavirus (Figure 2.2 A).

There was no evidence that susceptibility to FV3 (K = 0.432, Z = -0.801, P = 0.34) or the RC isolate (K=0.367, Z=-0.081, P = 0.59) was related to phylogenetic lineages. Thus, I proceeded with developing multiple regression models to identify host life history characteristics associated with infection prevalence. Overall, the AIC-candidate models for FV3 and RC isolates explained minimal variation (R^2 < 0.26) in infection prevalence (Tables 2.4, 2.5). Model-averaged weights for predictor variables suggested that breeding time (spring), aquatic index (temporary to semi-permanent wetlands), and hatching time (fast development) were associated with high susceptibility to ranavirus (Tables 2.7-2.8). The candidate models for the SM isolate explained substantial variation (R^2 > 0.79) in infection prevalence (Table 2.6). The most important variables associated with infection prevalence were aquatic index (temporary to semi-permanent wetlands), hatching time (fast development), and distance between host population and isolate location (Table 2.9).

DISCUSSION

I did not document a significant relationship between host phylogenies and susceptibility to ranavirus. Hoverman et al. (2011) reported a weak phylogenetic relationship with susceptibility to ranavirus, where Ranidae species appeared to be more susceptible to ranavirus compared to Ambystomatidae species. Given that my analyses included data from Hoverman et al. (2011), the disappearance of this trend was a consequence of the new species I tested. While some ranids that I tested were very susceptible to ranavirus (e.g., common frog; *Rana temporaria*) others had moderate to low susceptibility (e.g., crawfish frog; *Lithobates areolata*). Also, some ambystomatid species that I tested were very susceptible to ranavirus (e.g.,

northwestern salamander; *Ambystoma gracile*), when other were not (e.g. small mouthed salamander; *A. texanum*). The variation in susceptibility among species within families does not support the hypothesis of a co-evolutionary history between amphibian families and ranaviruses.

Phylogenetic signals for pathogen susceptibility have been documented. For example, monkey pox virus (Poxviridae) and canine distemper virus (Paramyxoviridae) infect and cause disease in multiple mammalian species over large geographic distances (Kamilar and Cooper 2013). Coevolution of ranaviruses with hosts has been reported (Losos 2008, Kamilar and Cooper 2013, Winter et al. 2013). Concordance of tiger salamander and ATV phylogenies and an increase in MHC Class I alleles in common frog populations exposed previously to an FV3-like ranavirus supports the hypothesis of a co-evolutionary history between ranaviruses and host populations (Storfer et al. 2007, Teacher et al. 2009). If the mechanisms that drive susceptibility to ranavirus were similar among species, it is likely that a phylogenetic signal would have been detected. Within amphibian families, there is tremendous variability in host species characteristics (Figure 2.2). Thus, host characteristics may be more important than phylogeny in affecting susceptibility to ranavirus.

I documented that amphibian hosts with fast developing larvae and that bred in temporary to semi-permanent wetlands were most susceptible to ranavirus, which Hoverman et al. (2011) documented as well. Representative species with high susceptibility and fast development included couch's spadefoot, ornate chorus frog, and wood frog, whereas species with low susceptibility and slow development included the marbled and Jefferson salamanders. Fast development is typical of species inhabiting ephemeral aquatic systems and an adaptation to avoid desiccation (Altig and Johnston 1989, Altig and McDiarmid 2007, Warkentin 2011). The greater susceptibility to ranavirus in species with fast development may be a consequence of

reduced immune response due to allocation of energy resources toward fast growth (Poorten and Kuhn 2009, Morales et al. 2010, Warne et al. 2010, 2011). Alternatively, mitotic cell division is accelerated in tadpoles during development (Goin et al. 1968, Godeau et al. 1986, Mueller et al. 2011); thus, species that develop faster likely have greater rates of cell division. Rapid division of suitable host cells facilitates replication by ranaviruses (Mao et al. 1996, Bollinger et al. 1999, Marsh et al. 2002).

Species that use temporary or semi-permanent habitats (e.g., gopher frog, Couch's spadefoot) tended to be more susceptible than species that used permanently flooded habitats (e.g., American bullfrog, striped newt). Habitats with short hydroperiods probably have fewer reservoirs for ranavirus (e.g., turtles, fish; Chapter III). Additionally, it is likely that ranavirus virions become inactivated after a few months of dry conditions at a breeding site (Nazir et al. 2012). Thus, species that inhabit temporary or semi-permanent wetlands likely have a lower probability of encountering ranavirus in the environment compared to species that use permanent wetlands. Given that ranaviruses and amphibian hosts can coevolve (Storfer et al. 2007), species that are exposed less frequently to ranavirus over evolutionary time presumably have fewer opportunities for coevolution, which may lead to reduced immune response during infection (Anderson and May 1979, May and Anderson 1979, Connell 1980, Liow et al. 2011, Blaustein et al. 2012).

I documented that species that breed during spring (e.g. wood frogs, common frogs) had greater susceptibility to the RC isolate than summer breeding species. It is possible that this result is correlative because several high susceptible species that breed during spring also breed in temporary wetlands and have fast development. This result may be an artifact of experimental design. My experiments were performed at room temperature (22^{o}C), which is warmer than

average water temperature during spring in Tennessee (15^{o}C, Schmutzer et al. 2008). Warm water temperature may act as a stressor on spring breeding species (Rojas et al. 2005, La Fauce et al. 2012), and increase susceptibility to ranavirus. Tadpole development also increases at warmer temperatures (Herreid and Kinney 1967, Harkey and Semlitsch 1988, Kuan and Lin 2011), which may facilitate ranavirus replication resulting in rapid host cell division. Given the spring breeding relationship was only observed with the RC, the aforementioned mechanisms might interact with unique viral properties (e.g., optimal replication temperature) that make this isolate more virulent at warmer temperatures.

For the SM isolate, susceptibility increased as distance between the host species population and isolate location increased. This trend supports the coevolution hypothesis, where populations that are located near an isolate have greater opportunity for evolution of immune function, hence would experience reduced susceptibility compared to distant populations (Liow et al. 2011, Blaustein et al. 2012). These results have conservation implications regarding the potential for pathogen pollution if ranavirus is transported over large geographic distance and released into a naïve population (Jancovich et al. 2005, Picco and Collins 2008). I did not test for this relationship with FV3 because it has been replicated under laboratory conditions for over 50 years which could have produced changes in infectivity (Pearman et al. 2004, Schock et al. 2008, Hoverman et al. 2010, Hoverman et al. 2011b). Also, I did not test for a distance relationship for the RC isolate because this ranavirus was isolated from a ranaculture facility where origin of the captive frogs was unknown.

I documented that susceptibility to the ranaculture isolate was greater than FV3 for many amphibian species, which supports findings by Hoverman et al. (2011). Greater infection prevalence and pathogenicity of the RC isolate may be related to evolution of virulence in this

isolate (Hoverman et al. 2011). Rapid transmission among hosts with competent immune systems (i.e., post-metamorphic American bullfrogs) may result in rapid evolution in captive facilities. Several other studies have documented greater pathogenicity of ranaviruses isolated from amphibians in captive facilities or bait stores (Majji et al. 2006, Storfer et al. 2007, Miller et al. 2008).

The pathogenicity of ranaviruses may differ among isolates based on the molecular array of the isolate, particularly related to the presence or absence of genes that facilitate viral infection (Chen et al. 2011, Chinchar et al. 2011b, Andino et al. 2012, Grayfer et al. 2012), and the level of immune response of the species tested (Robert et al. 2005, Morales et al. 2010, Robert and Chinchar 2012). According to Chen et al. (2011), the capacity of infection of a ranavirus isolate is directly related to its capacity to suppress the host translational control and down regulation of interferon that results in the shutdown of the cell translational machinery, and that inhibit the capacity of the virus to replicate. According to the authors, ranavirus isolates with the capacity to fully suppress the host interferon down regulation, are more infectious than isolates that lack that capacity. This concept is used by Jancovich et al., (2011) to describe the low infectivity of the FV3 type species isolated by Granoff (1965). According to the authors, this isolate only encodes a truncated version of the gene capable to suppress the host interferon down regulation, making it less virulent than other ranavirus isolates that have full capacity of suppression. This concept suggests that the differences in infectivity of the isolates used during this experiment can be the result of molecular dissimilarities among the isolates, more than the number of passages of the isolate as suggested by other authors (Pearman et al. 2004, Schock et al. 2008, Hoverman et al. 2010, Hoverman et al. 2011b).

My results indicate that host species characteristics are related to susceptibility to ranavirus. Species with tadpoles that develop rapidly and inhabit temporary to semi-permanent wetlands are at greatest risk, including species of conservation concern like the gopher frog (L. capito; endangered), or the boreal toad (*A. boreas*; threatened), as well as species of least concern like the wood frog (*L. sylvaticus*), or the southern leopard frog (*L. sphenocephala*). The capacity to identify the risk level of the species and the natural history traits related to ranaviral disease susceptibility could help us identify species and areas of greatest concern and allow us to focus conservation, surveillance, and management efforts more effectively. Furthermore, species identified as highly susceptible, could be used as models to identify the effects that ranavirus diseases could have in amphibian populations. Risks analyses that simulate population dynamics of highly susceptible species in the presence of ranavirus are currently being conducted by the University of Tennessee Center for Wildlife Health in collaboration with the national institute for mathematical and biological synthesis. These simulations will allow us to visualize the effects that the introduction of ranaviral diseases in the ecosystem could have in highly endangered species with extremely narrow distributions like the Mississippi gopher frog (*L. sevosa*), or in species of least concern with wide distributions like the wood frog (*L. sylvaticus*; Earl et al.unpublished data).

Chapter III

SUSCEPTIBILITY OF FIVE FISH AND THREE TURTLE SPECIES TO THREE DIFFERENT RANAVIRUS ISOLATES FROM DIFFERENT ECTOTHERMIC CLASSES

Ranaviruses have been associated with mortality of lower vertebrates around the world. Frog Virus 3 (FV3)-like ranaviruses have been isolated from different ectothermic vertebrate classes; however, few studies have demonstrated whether this pathogen can be transmitted among classes. Using FV3-like ranaviruses isolated from an amphibian (*Lithobates catesbeianus*), turtle (*Terrapene carolina carolina*) and fish (*Scaphirhynchus albus*), I tested for the occurrence of interclass transmission (infection prevalence) and relative susceptibility (percent mortality) for five fish and three turtle species exposed to each of these isolates. Exposure was administered via water bath (10^3 PFU/mL) for three days and survival was monitored for 28 days. Soft-shelled turtles (*Apalone ferox*) experienced no mortality but 10% and 20% of individuals were infected by the turtle and fish isolates, respectively. Similarly, 5% of Mississippi map turtles (*Graptemys pseudogeographica kohni*) were subclinically infected with the turtle isolate at the end of the experiment. Channel catfish (*Ictalurus punctatus*) experienced 5% mortality when exposed to the turtle isolate, while mosquito fish (*Gambusia affinis*) experienced 10% mortality when exposed to the turtle and amphibian isolates, and 5% when exposed to the fish isolate. My results demonstrate that interclass transmission of FV3-like ranaviruses is possible. Although substantial mortality did not occur in my experiments, the occurrence of low mortality and subclinical infections suggest that fish and chelonians may function as reservoirs for FV3-like ranaviruses. Additionally, my study is the first to report of transmission of FV3-like ranaviruses between fish and chelonians.

INTRODUCTION

Transmission of viruses among taxonomic classes (hereafter referred to as interclass transmission) is uncommon. Viral infection is a complex process that involves several steps and exploits a variety of cellular activities, ranging from endocytosis of the virion throughout the cell wall, to its importation into the cell nucleus (Su et al. 2008, Cronin et al. 2010, Jackson et al. 2010, Paull et al. 2012). The first and perhaps quintessential challenge the virus has to overcome after entering a new host is its replication. Once inside the new cell, the virus has to uncoat, transport its genetic materials to the appropriate cellular compartment, gather all the necessary replication machinery, produce copies of its genome and virion components, and package the genome into the capsids (Webby et al. 2004, Acheson 2007). If a virus successfully replicates in the new host cell, there are other obstacles that limit it from colonizing its new host. The virus must exit the cell (i.e., exocytosis or lysis of the cell), overcome or avoid immediate host immunological response, infect other cells quickly, and be shed from the host so transmission can occur (Webby et al. 2004, Bandin and Dopazo 2011, Crispe et al. 2011, Starick et al. 2011).

This complex process of host colonization makes interclass transmission unlikely in most cases. However, several viruses have found the way to overcome these obstacles, and examples of viruses transmitting between species have been recorded (Webby and Kalmakoff 1998, Keesing et al. 2010, Boelle et al. 2011, Swayne 2011). For example, some large dsDNA viruses, such as members of the family Iridoviridae, are known to infect multiple species (e.g., Hoverman et al. 2011). Iridoviruses enter the cell carrying start-up proteins that can be used as templates to initiate genome replication and protein production, facilitating the colonization of the host cell (Chinchar 2002, Chinchar et al. 2011a). The relative independence from its host and the high degree of conservation of the major capsid proteins, allows iridoviruses to successfully infect a

large variety of vertebrate and invertebrate hosts. Currently, five genera within Iridoviridae are recognized (King et al. 2012): two genera (Iridovirus and Chloriridovirus) infect arthropods (Camazine and Liu 1998, Hunter et al. 2001, Marina et al. 2003, Gregory et al. 2006), two genera (Lymphocystivirus and Megalocytivirus) infect fish (Sudthongkong et al. 2002b, Palmer et al. 2012, Rimmer et al. 2012, Waltzek et al. 2012), and one genus (Ranavirus) has been isolated from amphibians, fish and reptiles (Chinchar et al. 2009a, Cinkova et al. 2010, Vesely et al. 2011, Nazir et al. 2012, Robert and Chinchar 2012).

Ranaviruses have been associated with disease and mortality of numerous lower vertebrate species, including amphibians, fish and reptiles, and are considered a pathogen of ecological and economic importance (Chinchar 2002, Keesing et al. 2010, Robert and Chinchar 2012, Gray and Miller 2013). Currently, the international committee on taxonomy of viruses recognizes six species of ranaviruses (King et al. 2012). Three of the species infect fish exclusively such as the epizootic hematopoietic necrosis virus, European catfish virus, and Santee-cooper ranavirus; (Bigarre et al. 2008, Chinchar et al. 2009a, Whittington et al. 2010, Jensen et al. 2011a, Vesely et al. 2011), while the other species (Frog Virus 3, FV3; Ambystoma tigrinum virus, ATV; Bohle iridovirus, BIV) have been isolated most frequently from amphibian hosts, but might infect and cause disease in other ectothermic vertebrates. For example, ATV is known to cause high mortality in tiger salamanders (Jancovich et al. 2003a, Collins et al. 2004), and has been reported to cause infection in the largemouth bass (*Micropterus salmoides*, Picco et al. 2010). Also, BIV was originally isolated from an amphibian (Speare and Smith 1992, Cullen et al. 1995, Cullen and Owens 2002, Weir et al. 2012), but can infect fish and turtles (Moody and Owens 1994b, La Fauce et al. 2012). Recently, transmission of FV3-like ranaviruses was

demonstrated in fish (Jensen et al. 2009, Jensen et al. 2011a, Bayley et al. 2013) chelonians (Johnson et al. 2010), and multiple amphibian species (Hoverman et al. 2011).

Despite these findings, the host range of FV3-like ranaviruses remains unclear, especially with North American fish and chelonian species (Gray et al. 2009). Also, the possibility of interclass transmission of FV3-like ranaviruses has not been investigated extensively. My objective was to determine if three different FV3-like ranaviruses, isolated from hosts of three different ectothermic classes (amphibian, turtle, and fish) were able to cause infection and mortality in fish and turtle species known to coexist with amphibians or that are important to the aquaculture industry in North America. If interclass transmission is possible, fish and turtles may be important reservoirs of FV3-like ranaviruses (Gray et al. 2009), particularly in habitats where amphibians are not present yearlong.

METHODS

Ranaviruses and Hosts

The FV3-like ranaviruses were isolated from a morbid pallid sturgeon (*Scaphirhynchus albus*) in Missouri USA (T. Waltzek, unpubl. data), eastern box turtle (*Terrapene carolina carolina*) in Kentucky USA (Ruder et al. 2010), and American bullfrog (Lithobates catesbeianus) in Georgia USA (Miller et al. 2007). I tested five fish species: tilapia (*Oreochromis niloticus niloticus*), channel catfish (*Ictalurus punctatus*), mosquito fish (*Gambusia affinis*), bluegill (*Lepomis macrochirus*), and fathead minnow (*Pimephales promelas*). All fish species were fingerlings (ca. 5 – 10 cm length), and were obtained from commercial hatcheries (Table 3.1). Fish were reared from fry in independent outdoor concrete troughs, with no contact with other

species, no heating, and constant water flow. Upon arrival to the laboratory, a random sample of five individuals was humanely euthanized and tested for ranaviral infection (all results were negative). Prior to the start of the experiments, fishes were acclimated in the laboratory for a week in separate 1200-L tanks with flow-through, de-chlorinated water (20 gallons/second) at 25°C with 12:12 day: night photoperiod. During the acclimation period, fishes were fed daily a commercial high protein fish food (TetraMin®, Blacksburg, VA) ad libitum.

I tested three chelonian species; Florida soft-shelled turtle (Apalone ferox), eastern river cooter (*Pseudemys concinna*), and Mississippi map turtle (*Graptemys kohni*). Turtles were purchased as hatchlings (approximately 5 cm in average) from commercial retailers (Table 3.1). All species were raised in captivity and in isolation prior to shipment to the University of Tennessee. Turtles were housed under identical conditions as fish except floating platforms were added to 1200-L and specialized lamps were provided for thermal and UV exposure (Zoo Med Powersun UV Self-Ballasted Mercury Vapor UVB Lamp ®). A random sample of five individuals per species was euthanized to verify individuals were not infected with ranavirus prior to experimentation (all tested negative). Turtles were fed live crickets and bloodworms once a day ad libitum.

Fish Challenges

Each experimental trial consisted of four treatments with 20 replicate fish per treatment, totaling 80 experimental units; treatments were the three ranavirus isolates and a negative control. Eighty fish were randomly selected from the 1200-L tank and placed individually in 4-L (17.7 cm2) tubs, filled with 2 L of de-chlorinated aged tap water, and placed on 122 x 244 cm shelving units. Prior to adding the fish, each container was randomly assigned to a viral or

control treatment in a randomized block design, with two shelf heights as the blocking variable. Viral treatments were inoculated with 10^3 PFU/mL of the appropriate virus isolate, and the controls were inoculated with same quantity of virus-free media (i.e., minimum essential media, MEM Eagle). This viral titer is known to be sufficient to induce ranaviral disease in fish (Moody and Owens 1994b, Grizzle et al. 2002, Gobbo et al. 2010). Given that fish were negative for ranavirus at the beginning of each experiment, they did not contain active infections and the inoculations likely represented a first-time exposure to the pathogen, which is standard in ranavirus-challenge experiments (Jensen et al. 2011a, Jaramillo et al. 2012).

During the experiments, fish were fed commercial high protein food every day at a ratio of 3% of body mass, which is sufficient for normal growth and development (Budy et al. 2011). The amount of food required was calculated based on the body mass of a separate sample of five non-experimental fish that were treated identical to the controls. Fish were monitored twice daily for survival and morbidity. Dead individuals were removed from their containers, necropsied, and any gross signs of ranaviral infection recorded. Fish that exhibited morbidity consistent with ranaviral disease (i.e., petechial hemorrhages, edema, and loss of equilibrium) for >24 hours during the experiment were humanely euthanized. Water was changed (100% of volume) every three days to maintain water quality during the experiment (Hoverman et al. 2010). The duration for all trials was four weeks (28 calendar days), which is sufficient duration for morbidity to be observed from ranavirus infection (Jensen et al. 2009, Jensen et al. 2011a, Jaramillo et al. 2012). At the end of each experiment, all surviving individuals were humanely euthanized by immersion in benzocaine hydrochloride diluted in 90% ethanol at a 5 ml/L dosage until cessation of ventilating.

Turtle Challenges

The turtle experiments followed the same procedures as the fish challenges with three exceptions. First, the turtles were housed in 15.5-L containers (41.6 x 28.6 x 18.7 cm), with 2 L of de-chlorinated aged tap water (approximately 3 cm depth). This amount of water was sufficient for the turtle to be fully immersed, while maintaining its head above water. Second, during the experiments, turtles were fed two live crickets a day, which is sufficient for normal growth and development (Teece et al. 2001). Lastly, individuals that exhibit gross signs of ranaviral disease (e.g., cutaneous abscessation, oral ulceration or abscessation, respiratory distress, anorexia, and lethargy; Allender et al. 2006, Johnson et al. 2006) and survivors at the end of the experiment, were humanely euthanized via intravenous injection of sodium pentobarbital at a 60-100 mg/kg dose. All procedures followed approved University of Tennessee IACUC protocol #2052.

Ranavirus Testing

Genomic DNA (gDNA) was extracted from a tissue homogenate of the kidney and liver collected during necropsy using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). I used a QubitTM fluorometer and the Quant-iTTM dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA). Real-time PCR (qPCR) was used to verify presence or absence of ranavirus. The qPCR procedure was following that of Picco et al. (2007). All samples were run in duplicate and an individual was declared infected if the qPCR cycle threshold (CT) was <30 for both samples. The CT was determined for our PCR system (ABI 7900Fast Real-Time PCR System; Life Technologies Corporation,

Carlsbad, CA) by developing a standard curve using known quantities of virus. Four PCR controls were used for reference: positive control, negative control, FV3 control, and water.

Statistical Analyses

I summarized the results as individuals that died and were infected (case mortality), survived and were infected (subclinical infection), and died and were not infected (natural mortality). For each species, I tested for the difference in case mortality and infection prevalence among the ranavirus isolates using a G-test of maximum likelihood (Sokal and Rohlf 1995). All analyses were performed using SAS® 9.3 (SAS 2012) at $\alpha = 0.05$.

RESULTS

Two fish species experienced case mortality: channel catfish and mosquito fish (Figure 3.1). The channel catfish experienced 5% mortality when exposed to the fish isolate, while mosquito fish experienced 10%, 10%, and 5% mortality when exposed to the turtle, amphibian, and fish isolates, respectively. No statistical differences were detected in case mortality (G =5.71, 12 d.f., P =0.28) or infection prevalence (G =18.935, 12 d.f., P = 0.13) among the three isolates. Catfish died between 16 and 24 days post-exposure, while mosquito fish began to die after 4 days post-exposure to the virus.

No deaths were documented in turtles exposed to ranavirus; however, infection occurred in two species (Figure 3.2). Ten and 20% of soft-shelled turtles were infected after exposure to the turtle and fish isolates, respectively. The Mississippi map turtle experienced 5% infection

when exposed to the box turtle isolate. No statistical differences were detected in infection prevalence (G =7.32, 12 d.f., P = 0.19) among the three isolates.

DISCUSSION

My study documented two new cases of interclass transmission: (1) transmission of a FV3-like ranavirus isolated from a fish to a turtle species, and (2) transmission of a FV3-like ranavirus isolated from a turtle to a fish species. I also documented transmission of a FV3-like ranavirus isolated from an amphibian to a fish species, which has been reported by others (e.g., Bang Jensen et al. 2009, 2011; Gobbo et al. 2010; Picco et al. 2010). These results provide additional evidence that FV3-like ranaviruses can be transmitted among ectothermic vertebrate classes.

I documented 5% mortality of channel catfish exposed to the turtle isolate, and 5 – 10% mortality of mosquito fish exposed to fish, turtle or amphibian isolates. Although this level of mortality is low, these results suggest that ranavirus could impact aquaculture production and profits (Paperna et al. 2001, Sudthongkong et al. 2002a, Jensen et al. 2011b, Vesely et al. 2011). Jensen et al. (2011b) reported that ranaviruses were a concern to aquaculture industry in the European Union, and the occurrence of subclinically infected individuals in international fish trade could result in the emergence of ranavirus. Production of channel catfish and mosquito fish are major industries in the United States (Mischke et al. 2013, Torrans et al. 2013). Additionally, mosquito fish are commonly released into natural aquatic systems containing native populations of ectothermic vertebrates (Griffin and Knight 2012, Samidurai and Mathew 2013). The fact that

mosquito fish can be subclinically infected with fish and amphibian ranaviruses is a major conservation concern.

The species of ranaviruses that are found exclusively in fish hosts (i.e., epizootic hematopoietic necrosis virus, European catfish virus, and Santee-Cooper ranavirus) are known to cause significant morbidity and mortality in several fish species around the world (Bigarre et al. 2008, Picco et al. 2010, Whittington et al. 2010, Jensen et al. 2011b, Vesely et al. 2011). The ranavirus BIV also has been shown to cause significant mortality in barramundi; *Lates calcarifer*, (Moody and Owens 1994b). However, FV3-like and ATV ranaviruses appear to cause subclinical infections and low mortality in fish (Bang Jensen et al. 2009, 2011; Gobbo et al. 2010, Picco et al. 2010). The reduced susceptibility of fish to ATV and FV3-like ranaviruses could be a result of host specificity for cell entry and replication, or an inability to bypass the fully functional immune system of fish (Grayfer et al. 2012).

The low susceptibility of the turtles that I tested to ranavirus was unexpected, as cases of ranaviral infection and disease have been reported in at least 11 tortoise and box turtle species (Marschang et al. 1999, De Voe et al. 2004, Benetka et al. 2007, Johnson et al. 2007, Johnson et al. 2010, Marschang 2011), red-eared slider turtle (Johnson et al. 2006, Johnson et al. 2010) and Chinese soft-shelled turtle (Chen et al. 1999) in both natural and laboratory environments (Chen et al. 1999, De Voe et al. 2004, Allender et al. 2006, Johnson et al. 2008). However, most of these reports were diagnostic cases on a single individual or challenge experiments via intraperitoneal injection, which is an unrealistic transmission route (Gray et al. 2009). Population-level die-offs have been documented in eastern box turtles (Farnsworth and Seigel 2013); however, the effects of FV3-like ranaviruses on chelonians remain unclear and need further study. If future testing demonstrates low susceptibility of chelonians to ranavirus, host

specificity or inability to bypass the immune system may be mechanisms governing low ranavirus infection (Grayfer et al. 2012). Low susceptibility to FV3-like ranaviruses in fish and turtles compared to amphibians also may be a consequence of development rate. As seen in Chapter II, FV3-like ranaviruses appear to thrive in hosts that develop rapidly (Hoverman et al. 2011). Thus, the difference in susceptibility among ectothermic vertebrate classes could be a result of host developmental rates.

Lastly, these susceptibility results likely reflect a best-case scenario inasmuch as my experiments were conducted under controlled conditions with ad libitum food. Additionally, factors that contribute to ranavirus emergence such as density dependent transmission were controlled. In wild or captive populations, multiple infected and morbid individuals can be present, which might increase the likelihood of transmission to other ectothermic vertebrates, particularly those that predate (e.g., fish) or scavenge (e.g., turtles) other hosts.

My results demonstrate that fish and turtles could function as reservoirs for FV3-like ranaviruses and, through commercial trade, contribute to pathogen pollution. In the United States, 662 million tons of catfish (Hanson 2012) were produced in 2012 and according to the world chelonian trust (WCT 2013) between 2004 and 2005; 31.8 million turtles including 17,524,786 individual red-eared sliders (T. scripta) were sold in the United States. My results suggest that fish and turtles infected with ranavirus should be included in the World Organization for Animal Health (OIE) standards for notifiable diseases (Schloegel et al. 2010). Currently, amphibians infected with ranaviruses are the only taxonomic group listed in the OIE regulations (Schloegel et al. 2010).

Although my results showed that some fish (catfish and mosquito fish) and turtle (soft-shelled and Mississippi map) are suitable hosts for FV3-like ranaviruses, additional research is needed on other species in North America. Additionally, experiments are needed to determine if an infected individual of one class can transmit the virus through water to a different class (Chapter III). The capacity of fish and turtle species to transmit the ranavirus to highly susceptible hosts that inhabit aquatic environments seasonally (e.g., amphibians) will help us understand the re-occurrence of outbreaks in ecosystems with fluctuating species composition (Pearman and Garner 2005, Teacher et al. 2010). This information could be essential for the planning and execution of conservation strategies for areas that exhibit recurrent ranavirus outbreaks, as well as the identification of areas with risk of ranaviral disease.

Chapter IV

TRANSMISSION OF RANAVIRUS BETWEEN ECTOTHERMIC VERTEBRATE HOSTS

Transmission is an essential process than contributes to the survival of pathogens. Ranaviruses are known to infect different classes of lower vertebrates including amphibians, fishes, and reptiles. Differences in the likelihood of infection between or among ectothermic vertebrate hosts could explain the successful yearlong persistence of ranaviruses in aquatic environments. The goal of this study was to determine occurrence of transmission of a Frog Virus 3-like ranavirus among three species from different ectothermic vertebrate classes: Cope's gray treefrog (*Hyla chrysoscelis*) larvae, mosquito fish (*Gambusia affinis*), and red-eared slider (*Trachemys scripta*). I housed individuals previously exposed to the FV3-like ranavirus with naïve individuals in containers divided by plastic mesh screen to permit water flow between subjects. My results showed that infected gray treefrog larvae were capable of transmitting ranavirus to naïve larval conspecifics, turtles, and fish (70%, 40%, and 10% infection, respectively). Also, infected turtles and fish transmitted ranavirus to 50% and 20% of the naïve gray treefrog larvae, respectively. Nearly all of infected amphibians experienced mortality, whereas infected turtles and fish did not die. My results demonstrate that ranavirus can be transmitted through water among ectothermic vertebrate classes. Moreover, fish and reptiles might serve as reservoirs for ranavirus given their ability to live with subclinical infections. Subclinical infections of ranavirus in fish and aquatic turtles could contribute to its persistence, especially when highly susceptible hosts like amphibians are absent as a result of seasonal fluctuations in relative abundance.

INTRODUCTION

The persistence of an infectious disease in the environment is directly related to the availability of suitable hosts and the likelihood of pathogen transmission. In aquatic environments, pathogens can be transmitted between hosts via direct contact, predation, or by indirect waterborne contact (Haydon et al. 2002, Brunner et al. 2007). The route and magnitude of transmission depends on host density and environmental factors such as water temperature and pH (Haydon et al. 2002, Brunner et al. 2007, Breban 2013). When host densities are high, direct transmission via close contact such as bumping or fighting can occur. Conversely, when host densities are low or fluctuating in aquatic environments, indirect transmission through water may be most efficient (Brunner et al. 2007, Breban 2013). Most pathogens that inhabit environments with fluctuating host densities are able to infect host species with different levels of susceptibility (Haydon et al. 2002, Woolhouse et al. 2005).

Ranaviruses are large DNA viruses from the Iridoviridae family, a diverse group of viruses known to infect multiple lower vertebrate hosts including amphibians (Duffus et al. 2008, Schock et al. 2008, Schock et al. 2009, Hoverman et al. 2010, Hoverman et al. 2011b), fish (Moody and Owens 1994a, Jensen et al. 2009, Whittington et al. 2010, Jensen et al. 2011b), and reptiles (Hyatt et al. 2002, De Voe et al. 2004, Johnson et al. 2008, Marschang 2011). High variation in susceptibility of amphibians, fish and chelonians to ranaviruses has been reported (Ariel and Owens 1997, Johnson et al. 2006, Whittington et al. 2010, Allender et al. 2011, Hoverman et al. 2011b, Jensen et al. 2011a). Differences in host susceptibility to ranaviruses create an ideal scenario for the pathogen to move between hosts, utilizing highly susceptible species for rapid viral replication and low susceptible hosts that can maintain the pathogen (Paull et al. 2012). Reservoirs composed of subclinically infected hosts might explain the yearlong

persistence of ranaviruses in the environment (Gray et al. 2009). Many communities where ranaviruses emerge contain multiple species from different ectothermic vertebrate classes (e.g., Farnsworth and Seigel 2013).

Given the variability in susceptibility to ranavirus for host species within and among ectothermic vertebrate classes, presumably one class could function as a reservoir of ranavirus for another class. In experiments that I performed in Chapter III, I demonstrated that interclass transmission was possible via exposure to ranavirus inoculum in water at a concentration of 10^3 PFU/mL, which is believed to be an environmentally relevant concentration during a die-off (Rojas et al. 2005). Although water bath exposure to a standard concentration of ranavirus inoculum is useful for controlled experiments, it may not be representative of natural transmission. Thus, my goal was to test whether interclass transmission of ranavirus was possible between ectothermic vertebrate classes using hosts as the mechanism for transmission. These results will provide insight into the likelihood of transmission between vertebrate classes and the possible role of different hosts functioning as reservoirs or amplification hosts for ranavirus.

METHODS

To determine the capacity of ranavirus transmission between ectothermic vertebrate classes (fish, reptiles and amphibians), I set up an experimental challenge between three sympatric species known to be susceptible to ranavirus infection: mosquito fish (*Gambusia affinis*, Chapter III), red-eared slider turtle (*Trachemys scripta elegans*; Gray et al. unpubl. data), and Cope's gray treefrog (*Hyla chryscocelis*; Hoverman et al. 2010). I used a Frog Virus 3

(FV3)-like ranavirus that was isolated from a pallid sturgeon (Scaphirhynchus albus) during a die-off in an aquaculture facility (T. Waltzek, University of Florida, unpublished data). All the species were known to be susceptible when exposed to this isolate, exhibiting 70%, 40% and 10% mortality Cope's gray tree frog tadpoles, red-eared slider hatchlings, and mosquito fish, respectively.

The mosquito fish used for the experiment were obtained as fingerlings (ca. 5-10 cm length) from a commercial hatchery and acclimated in the laboratory for a week prior to the experiment in a 1200-L tank with constant, flow-through water (20 gallons/second) at 26°C. Turtles were obtained as hatchlings (approximately 5 cm in average) from a commercial retailer (Turtle shack, Port Richey, FL) and acclimated for a week in a 1200 L tub with floating platforms and high power lights for basking. Maintenance and feeding protocols during acclimation were identical to Chapter III. Amphibian larvae were collected as egg masses from local wetlands and hatched and raised in 324 L wading pools. Acclimation maintenance and feeding protocols were identical to Hoverman et al. (2010).

To test transmission of the pathogen, I used 15.5-L containers divided in half by a 2000 µm plastic mesh (design adapted from Harp and Petranka 2006). Each container housed an individual of a different species on each side of the container. Containers were filled with 2 L of dechlorinated aged tap water (approximately 3.5 cm depth) sufficient for turtles to be fully immersed in water when maintaining their head above water to avoid drowning. Tubs were divided into control and virus treatments; viral treatments were inoculated with 10^3 PFU/mL of the pallid sturgeon isolate, and controls were inoculated with an equal quantity of virus-free media (i.e., minimum essential media, MEM Eagle). The challenge was designed as a factorial experiment using a randomized block design with shelf position serving as blocks. The tubs

were placed on four shelving units (122 x 244-cm) with four shelves per unit. I placed 20 tubs (5 per level) on each shelving unit, totaling 80 experimental units. Viral treatments were paired as follows: 1) exposed turtle and unexposed tadpole, 2) exposed tadpole and unexposed turtle, 3) exposed fish and unexposed tadpole, 4) exposed tadpole and unexposed fish, and 5) exposed tadpole and unexposed tadpole. An identical complement of paired control treatments were conducted.

For each experiment, tadpoles, fish and turtles were randomly collected from the acclimation tanks and assigned to a treatment (virus or control). The virus challenge was through water bath exposure for three days, sufficient duration to initiate infection in tadpoles (Hoverman et al. 2010, Haislip et al. 2011), fish (Moody and Owens 1994c, Jensen et al. 2009, Gobbo et al. 2010), and turtles (Johnson et al. 2006, Johnson et al. 2007). During the time of exposure, no food was dispensed to avoid unknown impacts on transmission. After the inoculation period and every three days thereafter, water was changed (100% of volume) to maintain water quality. Amphibian larvae were fed ground fish food (TetraMin®) at a ratio of 12% of their body mass every three days (Hoverman et al. 2010), and turtles and fish were fed high protein catfish pellets (TetraMin®, Blacksburg, VA) every other day at a ratio of 3% of their body mass sufficient for normal growth and development (Budy et al. 2011). Turtles were weighed before the beginning of the experiment to determine the amount of food required. To estimate the amount of food for the fish, I followed the same protocol used for the tadpoles in chapter III.

During the experiment, all individuals were monitored twice daily for survival and morbidity. Bodies of exposed individuals that died during the trial were left in the tub for three days after death to allow normal virus shedding post mortem. Conversely, unexposed individuals that died during the experimental trial were removed from the containers as soon as possible to

reduce decomposition, which can affect performance of the PCR (Blacksell et al. 2004). Removed individuals were necropsied and sections of liver and kidney were removed for virus identification by PCR analysis. Room temperature in the laboratory was maintained at 25°C and the photoperiod was set at 12:12 day:night to emulate natural conditions (Relyea and Werner 1999).

The duration of the experiment was four weeks (28 days), which is sufficient time to observe morbidity in infected individuals (Johnson et al. 2007, Hoverman et al. 2011b, Jensen et al. 2011a). At the end of the experiment, all surviving individuals were humanely euthanized and tested for ranaviral infection using real-time quantitative PCR (qPCR). Surviving amphibian larvae and fish individuals were euthanized by immersion in a solution of benzocaine hydrochloride diluted in 90% ethanol at a 5 ml/L dosage until cessation of ventilation. Turtles were euthanized by intravenous injection of sodium pentobarbital at a 60-100 mg/kg dose. All husbandry and euthanasia procedure followed approved University of Tennessee IACUC protocol # 2018.

I extracted genomic DNA (gDNA) from liver samples using the DNeasy Blood and Tissue Kit® (Qiagen Inc., Valencia, CA). I used a QubitTM fluorometer and the Quant-iTTM dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA). Real-time PCR (qPCR) was used to verify presence or absence of ranavirus. The qPCR procedure followed that of Picco et al. (2007). All samples were run in duplicate and an individual was declared infected if the qPCR cycle threshold (CT) was <30 for both samples. The CT was determined for our PCR system (ABI 7900Fast Real-Time PCR System; Life Technologies Corporation, Carlsbad, CA) by developing a standard curve using

known quantities of virus. Four PCR controls were used for reference: positive control, negative control, FV3 control, and water.

To determine the differences in mortality and infection prevalence among species and isolates, I use two 2-way ANOVA's (Sokal and Rohlf 1995). I used Person's correlation coefficient to quantify the linear relationship between infected individuals and infected individuals that died across virus treatments and species. All analyses were performed using SAS® 9.3 (SAS 2012) at $\alpha = 0.05$.

RESULTS

Results showed that across all species ranavirus could be transmitted between hosts causing significant infection (F =9.79, 4 df, P =0.03) and mortality (F =14.09, 4 df, P =0.02) in naïve individuals housed with infectious individuals via water bath. Susceptibility of directly (prior exposure) and indirectly (by co-inhabitant) exposed individuals varied among host species. Amphibian larvae were the most susceptible species with 73% infection of individuals directly exposed to the ranavirus (Figure 4.1), and 40% infection of individuals exposed indirectly by other infected hosts. Ranavirus transmission from infected amphibian larvae to naive hosts was observed in 60% of conspecifics and 30% of turtles. Mortality of amphibian larvae indirectly infected by infectious turtles was 100%, but no mortality was observed for indirectly infected turtles (Figure 4.2). Interestingly, when housed with fish, direct infection of amphibians was 70%, but transmission of the pathogen to the naïve fish by amphibians was not observed.

After 28 days, 20% of directly exposed turtles were infected while 50% of amphibians that were housed with them became infected and died (Figure 4.1), suggesting that at least 30%

of turtles cleared the virus before the end of the experiment. Directly exposed fish showed low susceptibility to the ranavirus (20% infection, 0% mortality). These individuals transmitted the virus to 10% of the co-inhabitant amphibian larvae.

The average difference in time of death between the directly exposed individuals and their indirectly exposed counterpart was 6 ±2.70 days (Figure 4.3). The actual time between infections cannot be determined as transmission of the pathogen from the infected host could be initiated pre-mortem. Time of infection and mortality of amphibians infected by other hosts could not be identified because no deaths of reptiles or fish were observed, but average indirect mortality took 2.35 ±0.85 days longer than direct mortality (Table 4.1).

DISCUSSION

The main objective of this experiment was to determine if ranavirus could be transmitted between ectothermic vertebrate classes. Infected mosquito fish and red-eared sliders were able to transmit ranavirus to Cope's gray treefrog tadpoles and cause 10% and 50% mortality, respectively. Infected gray treefrog tadpoles were able to transmit ranavirus to red-eared sliders (30% infection), but none of the turtles died after 28 days. Infected gray treefrog tadpoles were unable to transmit ranavirus to mosquito fish. Alternatively, mosquito fish may have become infected when exposed to infectious tadpoles but cleared the virus within 28 days when the experiment ended and surviving individuals were tested for infection. Infected gray treefrogs efficiently transmitted ranavirus to conspecifics (60% infection); all infected conspecific died.

These results demonstrate that interclass transmission of ranavirus is possible through water by virion shedding from an infected individual. Previous studies (e.g., Bayley et al. 2013,

Chapter III) have inferred interclass transmission by exposing a host in one vertebrate class to a ranavirus isolated from different vertebrate class. My results also suggest that larval amphibians might be amplification hosts as demonstrated by high infection prevalence and mortality; whereas, fish and turtles may function as reservoir species due to lower susceptibility (Paull et al. 2012). My experiment was conducted with only one species from each ectothermic vertebrate class. Experiments are needed with additional species to determine if this this trend holds.

Levels of mortality observed in my study were slightly lower than individual species challenge studies performed by others. For example, Hoverman et al. (2011) reported 80% mortality of Cope's gray treefrog tadpoles exposed to a FV3-like ranavirus inoculum in a water bath. I found that 10% of mosquito fish became infected with half of those individuals dying when exposed to the same virus isolate (Chapter III). Gray et al. (unpubl. data) found that 40% of red-eared sliders became infected and died when exposed to same isolate as my experiment. The differences in mortality rates may be a consequence of virion concentration in the water. The aforementioned studies exposed hosts to 10^3 PFU/mL of ranavirus, while unexposed individuals in my study were exposed to one individual that was previously exposed to ranavirus inoculum at 10^3 PFU/mL. Individuals exposed to the inoculum may not have become infected or perhaps shed virions at a concentration $<10^3$ PFU/mL when housed with a naïve individual. Dose dependency of ranavirus pathogenicity has been reported (Pearman et al. 2004, Brunner et al. 2005).

Although transmission of ranavirus from an infected to a naïve amphibian larvae via water bath has been documented previously (Harp and Petranka 2006), my results represent the first observation of high level of infection (60%) and mortality (50%) of individuals exposed to the pathogen solely by cohabitation with infected hosts. Harp and Petranka (2006) reported low

levels of infection (25%) and no mortality of naive wood frogs (*Lithobates sylvaticus*) after 111 hrs of cohabitation with infected conspecifics, attributing the low infection and lack of mortality observed to low levels of viral load shed by the moribund tadpoles. Although duration of cohabitation was longer in my study, Robert et al. (2011) demonstrated transmission of ranavirus could occur as quickly as 3 hours in a water bath.

The capacity of subclinically infected fish and turtles to transmit ranavirus to amphibians has important implications regarding the persistence of this pathogen in aquatic environments. Reports of ranavirus outbreaks, particularly affecting amphibian communities, have been well documented (Weng et al. 2002, Greer et al. 2005, Une et al. 2009a, Une et al. 2009b, Balseiro et al. 2010, Hoverman et al. 2012). In many cases, these outbreaks have been reported to be seasonally recurrent (Greer et al. 2005, Cunningham et al. 2007a, Teacher et al. 2010). Most reports of recurrent ranavirus outbreaks in amphibian communities describe high levels of disease and mortality during periods of time when amphibian larvae are highly abundant followed by an abrupt cease or a significant decrease of disease as the abundance of amphibian larvae decreases and larvae start leaving the aquatic environment after metamorphosis (Todd-Thompson 2010) During these periods when amphibian density is low or completely absent from the water bodies, ranavirus appears to be absent, but ranavirus prevalence can increase rapidly as soon as the next generation of amphibians returns to the aquatic ecosystems (Greer et al. 2005, Cunningham et al. 2007a, Teacher et al. 2010). It has been hypothesized that ranavirus can persist in aquatic environments via biological reservoirs (Gray and Miller 2013). Ranaviruses might persist in fish and aquatic turtles when the availability of highly susceptible hosts like amphibian larvae is reduced (Haydon et al. 2002, Brunner et al. 2004, Brunner et al. 2005).

According to Cronin et al. (2010), ideal reservoir species are those that can harbor subclinical infections of pathogens without suffering any impairment in their biological or ecological functions until the right conditions arrive and the pathogen can again be release into the environment where it can then invade new hosts more suitable for its replication. For this to occur, the pathogen must exhibit three basic characteristics (Anderson and May 1979, May and Anderson 1979, Cronin et al. 2010). First, it should display different levels of infectivity among host either by being able to infect different species at different rates or by infecting different ages or developmental stages of the same species at different rates. In the case of ranaviruses, differences in susceptibility ranging from low to high susceptibility has being described for amphibians species (Hoverman et al. 2010, Hoverman et al. 2011b) and life stages (Haislip et al. 2011) as well as for fish (Jensen et al. 2009, Gobbo et al. 2010, Jensen et al. 2011a) and reptiles species (Johnson et al. 2007, Johnson et al. 2008, Allender et al. 2009, Allender et al. 2011). Second, the pathogen must be able to be transfer efficiently among hosts. Ranaviruses have been reported to be able to transmit among hosts by contact (Brunner et al. 2007), consumption (predation or cannibalism; Brunner et al. 2007), and most commonly via water exposure (Jancovich et al. 2005, Cunningham et al. 2007b, Nazir et al. 2012). Third, host availability should fluctuate through time between high and low susceptible species. Because of the complex life cycle and breeding phenology of amphibians, fluctuations in abundance and composition of amphibian communities is common (Vignolia et al. 2007, Werner et al. 2007) .

These three characteristics of amphibian communities might facilitate the persistence of ranavirus. I hypothesize that ranaviruses persist at low prevalence in low susceptible hosts, such as aquatic turtles and fish, and emerge when highly susceptible hosts, such as many species of

larval amphibians, become abundant. Moreover, if low susceptible hosts are highly mobile, they may contribute to overland transport of ranaviruses.

More research is needed on the susceptibility of other ectothermic vertebrates, especially chelonians and fish, to understand the complex dynamics of ranaviruses in the environment throughout the year. Identification of amplification and reservoirs species will facilitate modeling of ranavirus transmission dynamics, and development of tools that could predict likelihood of ranavirus outbreaks. Knowledge of potential ranavirus reservoirs also could assist formulation of conservation strategies for areas where outbreaks have been documented. For example, removal of a fish reservoir might prevent an outbreak from occurring.

CHAPTER V

COMMUNITY COMPOSITION AFFECTS OUTCOME OF A RANAVIRUS OUTBREAK

The occurrence and outcome of a pathogen outbreak could be mediated by host susceptibility. In pathogens with multiple host species, community composition may play a role in transmission. For example, communities composed of highly susceptible species may experience greater mortality. Additionally, the outcome of an outbreak may depend on which species is initially exposed to the pathogen. My objective was to determine if the outcome of a ranaviral disease outbreak in an amphibian community was dependent on species composition and which species was initially exposed to the pathogen. I created two amphibian communities: (1) an Appalachian community composed of wood frog (*Lithobates sylvaticus*), upland chorus (*Pseudacris feriarum*), and spotted salamander (*Ambystoma maculatum*) larvae, and (2) a coastal plain community composed of gopher frog (*Lithobates capito*), upland chorus frog (*P. feriarum*), and southern toad (*Anaxyrus terrestris*) larvae. The experiment was conducted outdoors in 324-L mesocosms, and treatments consisted of one, all, or none of the species initially exposed to Frog Virus 3, the type species of Ranavirus. Mortality rates after 60 days depended on which species was initially exposed to the pathogen. In the Appalachian community, exposed wood frog tadpoles caused an outbreak of ranaviral disease in unexposed chorus frogs (40% mortality) and doubled mortality of spotted salamander larvae. In the coastal plains community, all species were able to cause outbreaks of ranaviral disease (>40% mortality) in syntopic unexposed species. My results demonstrate that amphibian community composition can affect ranaviral disease outcomes. Additionally, wood frog tadpoles may function as amplification species.

INTRODUCTION

The capacity of a host to transmit a pathogen is dependent on three main factors: the degree of contact the host has with other individuals or species, the degree of susceptibility of the host to the pathogen, and the amount of pathogen shed by the host (Paull et al. 2012). These three characteristics will determine the contribution of the host to pathogen persistence in the environment as well as transmission within the community. Often, these characteristics are species- or individual-specific, creating great variability in capacity of transmission among individuals within a community (Woolhouse et al. 1997, Haislip et al. 2011, Hoverman et al. 2011a). Individual or species variation in the ability to transmit a pathogen can be so pronounced that a small percent of individuals results in the majority of infections within the community (Thrall et al. 1995, Woolhouse 2011). This disproportional capacity of transmission observed in some individuals was described by Woolhouse et al. (1997) as the 20/80 rule of dispersion, where 20% of the individuals can be responsible for 80% of the infections in the population. These highly contagious individuals responsible for high levels of pathogen transmission are known as superspreading individuals (Woolhouse et al. 1997, Lloyd-Smith et al. 2005, Johnson and Paull 2011), and have the capacity to amplify (i.e., increase beyond normal boundaries) intraspecific transmission resulting in species-level outbreaks. For pathogens known to infect multiple host species, highly susceptible species can be superspreaders, which Paull et al. (2012) called amplification species.

Ranaviruses are an emerging pathogen (Gray and Miller 2013) that are known to infect multiple amphibian species with differences in susceptibility (Hoverman et al. 2011). In amphibian communities composed of multiple species, the presence of highly susceptible species might increase mortality of syntopic species, thereby resulting in a community-level outbreak.

Multiple species outbreaks of ranavirus have been observed frequently (Green et al. 2002, Greer et al. 2005, Gahl and Calhoun 2010, Todd-Thompson 2010). In eastern North America, many of these outbreaks are associated with vernal pool communities containing wood frogs (*Lithobates sylvaticus*; Greer et al. 2005, Gahl and Calhoun 2010). Wood frogs are known to be highly susceptible to ranavirus (Hoverman et al. 2011); thus, their presence may result in a disease hotspot, which are areas where there is a disproportionally high level of pathogen transmission and persistence (Paull et al. 2012). Disease hotspots can serve as source areas from which pathogens are dispersed to less infected areas across the landscape, thereby increasing pathogen survival and securing its persistence in the environment (Paull et al. 2012).

The highly variable levels of ranavirus susceptibility in amphibian species and the natural complexity of amphibian communities make this system ideal for studying the effects of community composition on disease outbreaks. My objective was to test for differences in percent mortality among ranavirus exposure treatments for two amphibian communities composed of hosts with different susceptibility. I hypothesized that if a highly susceptible species (e.g., wood frog or gopher frog, *L. capito*) were exposed to ranavirus, greater levels of mortality would be observed than if low susceptible species were initially exposed to the pathogen.

METHODS

To determine the capacity of transmission between different amphibian species in semi-natural conditions, I performed two aquatic mesocosm experiments during the summers of 2011 and 2012. Each experiment consisted of a different amphibian assemblage typical of the southeastern United States. In 2011, the community was composed of wood frog (*Lithobates*

sylvaticus), upland chorus frog (*Pseudacris feriarum*), and spotted salamander (*Ambystoma maculatum*) larvae, which is a typical community of the eastern United States at the latitude of Tennessee northward. These species are susceptible to ranaviruses with 95%, 40%, and 35% mortality, respectively, in single species challenge experiments (Hoverman et al. 2011). The second year of the experiment, the amphibian community was typical of the Coastal Plains in the southern United States: gopher frog (*L. capito*), upland chorus frog (*P. feriarum*), and southern toad (*Anaxyrus terrestris*), with mortality rates of 60%, 40%, and 40% respectively (Hoverman et al. 2011, Chapter II).

Individuals used in these experiments were collected as egg masses from non-experimental wetlands in eastern Tennessee, or acquired from researchers across the country. Egg masses were hatched and raised in 324-L outdoor culture pools at the University of Tennessee Joe Johnson Animal Research and Teaching Unit (JARTU). Pools were covered with 70% shade cloth lids that allow the larvae to experience natural diel temperature fluctuations and photoperiods; water temperature in the pools ranged between 29°C and 32°C during both experiments. To standardize the effects of development on susceptibility (Brunner et al. 2004, Haislip et al. 2011), I started experiments when anuran larvae were Gosner 25 – 30 stage (Gosner 1960) and salamander larvae were 1 – 2 months post-hatch.

Virus Exposure

Once the larvae reached the appropriate developmental stage, 250 larvae from each species (3 species = 750 individuals) were haphazardly collected from the outdoor mesocosms, moved to the laboratory, housed individually in 2-L containers filled with 1-L of de-chlorinated aged tap water, and placed on 122 x 244-cm shelving units separated by treatments. The five

treatments were: (1) all species exposed to virus, (2 – 4) three treatments where only one species was exposed to the virus, and (5) a negative control where none of the species were exposed to virus. Thus, treatment 1 = species 1 (exposed), species 2 (exposed), species 3 (exposed); treatment 2 = species 1 (exposed), species 2 (not exposed), species 3 (not exposed); treatment 3 = species 1 (not exposed), species 2 (exposed), species 3 (not exposed); treatment 4 = species 1 (not exposed), species 2 (not exposed), species 3 (exposed); and control. Individuals assigned to exposed treatments received (by direct water inoculation) 10^3 PFU/mL of FV3 and the unexposed treatments received the same quantity of virus-free MEM Eagle growth media. The FV3 isolate was originally cultured by Allan Granoff from clinically normal adult leopard frogs (L. sphenocephala) in Illinois, USA (Granoff et al. 1965). The virus used for these experiments was cultured and tittered by Dr. Rebecca Wilkes in the Department of Biomedical and Diagnostic Sciences in the College of Veterinary Medicine at the University of Tennessee. Water bath exposures lasted for three days, which is sufficient duration to initiate infection in exposed individuals (Hoverman et al. 2010). During the ranavirus exposure, individuals were kept in the laboratory at 25°C and exposed to a 12:12 day: night photoperiod. After the three-day exposure, larvae were transported to the mesocosm site and added to a corresponding mesocosm (i.e., larvae in containers were not split between mesocosms).

Mesocosms

Mesocosms were created in 324-L wading pools (n = 25, 5 per treatment) and covered with 70% shade cloth lids. Wading pools were arranged in a 5 x 5 grid on flat ground with approximately 1 m separation. To create semi-natural conditions in mesocosms, I conditioned the pools over six weeks (Relyea and Diecks 2008). In week one, the pools were filled with aged tap water. In week two, I added 97 g of dry leaf litter (primarily *Quercus* spp.), 8 g of rabbit chow as

an initial nutrient source, and 1 L of "green water" which had been aged for >2 years in 1200-L outdoor cattle tanks that contained natural growth of phytoplankton. In week four, I added a 50-mL container of *Daphnia* spp and mixed rotifers from Carolina Biological Supply (Burlington, NC). After two weeks, I translocated amphibian larvae from the laboratory (where individuals were inoculated) to the pools. To maintain the environment as natural as possible, I did not perform water changes during the experiments (Relyea and Hoverman 2008). To determine transmission I defined the following possible outcomes: (1) amplification; defined as when mortality of the unexposed species caused by indirect transmission from the exposed species was ≥ 2X greater than mortality caused by direct exposure under laboratory conditions (Paull et al. 2012), (2) outbreak; defined as when mortality of the unexposed species caused by indirect transmission from the exposed species was ≥ 40%, as define for diseases hotspots by Hoverman et al. (2012), and (3) transmission, defined as when the exposed species was able to cause mortality in the unexposed species (Gray and Miller 2013).

Data Collection

Larvae were monitored daily for survival; dead individuals were removed from the pool immediately and necropsied (see below). Floating platforms were added to mesocosms as individuals begin to metamorphose to prevent drowning. Larvae were removed from the pools when tadpoles had completely resorbed their tails and salamanders had resorbed their external gills. Metamorphs were deemed survivors and humanely euthanized using benzocaine hydrochloride (Chapter II). All procedures followed University of Tennessee IACUC protocol #2009. Experiments lasted for 8 weeks not including conditioning time for mesocosms.

All dead larvae were necropsied and a section of liver and kidney removed for virus testing. The remaining portion of the larva was placed in 10% neutral buffered formalin. In cases when the individual was very small (less than 1 cm total length), the specimen was cut in half (sagittal) and a one half of the body was used for virus testing while the remaining section was stored in formalin. To verify that dead individuals were infected with ranavirus, I ran real-time quantitative PCR. Genomic DNA (gDNA) was extracted from the liver and kidney homogenate using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). I used a QubitTM fluorometer and the Quant-iTTM dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen Corp., Carlsbad, CA, USA). The qPCR procedure followed that of Picco et al. (2007). All samples were run in duplicate and an individual was declared infected if the qPCR cycle threshold (CT) was <30 for both samples. The CT was determined for our PCR system (ABI 7900Fast Real-Time PCR System; Life Technologies Corporation, Carlsbad, CA) by developing a standard curve using known quantities of virus. Four PCR controls were used for reference: positive control, negative control, FV3 control, and water.

Analysis

To test for differences between mortality of individuals when directly exposed to the virus by inoculation I used two-way ANOVA. To determine differences in mortality values of species among treatments I used one-way ANOVA's and Levene's test to determine homogeneity of variance, when data was not normally distributed I used Kruskal-Wallis test with Wilcoxon scores (Sokal and Rohlf 1995, Dytham 1999). To estimate differences among groups I used Tukey's studentized range test and Bonferroni (Dunn) test (Zar 1996). All analyses were conducted using SAS 9.3 (SAS 2012) with an α=0.05.

RESULTS

Mortality of wood frog, chorus frog and spotted salamander larvae differed significantly among exposure treatments (Kruskal-Wallis X^2 = 25.48, 2 df, P = 0.01), and depended on which species was initially exposed to ranavirus inoculum in a water bath (hereafter referred to as directly exposed). Directly exposed wood frog larvae transmitted ranavirus and caused 43% and 12% mortality in chorus frog and spotted salamander larvae, respectively (Figure 5.1A). Directly exposed chorus frog larvae transmitted ranavirus and caused 72% and 3% mortality in wood frog and spotted salamander larvae, respectively (Figure 5.1B). Directly exposed spotted salamander larvae transmitted ranavirus and caused 18% and 24% mortality in wood frog and chorus frog larvae, respectively (Figure 5.1C). Community-level mortality (i.e., averaged among species) was 54%, 40%, and 16% when wood frog, chorus frog, and spotted salamander larvae were directly exposed to ranavirus, respectively. In the treatment when all individuals were directly exposed, mortality was 100%, 50% and 10% for wood frog, chorus frog, and spotted salamander larvae, respectively. All individuals that died tested positive for ranavirus infection, and there was no mortality in the control treatment.

Species mortality varied depending on if it was direct exposure to the ranavirus or if it was infected indirectly by a co-inhabitant species (Table 5.1). Wood frog mortality when exposed to the virus directly (100%) was higher than when infected indirectly by contact with directly exposed chorus frogs (72%), or spotted salamanders (18%,), mortality rates were significantly different among exposures (F =30.78, 2df, P =0.012, Figure 5.1 A). Chorus frog showed no significant differences in mortality (F =1.38, 2df, P =0.09, Figure 5.1 B) when exposed to the virus directly (44%) or when exposed indirectly by contact with directly exposed wood frogs (42%), or spotted salamanders (24 %,). Mortality of spotted salamanders was

significantly higher when exposed to the ranavirus indirectly by contact with directly exposed wood frogs (12%; Kruskal-Wallis X^2 =6.57, 2 df, P =0.03, Figure 5.1 C), than when exposed to the virus by direct inoculation (6%), or by indirect contact with directly exposed chorus frogs (4.0%).

I did not detect a statistical difference in mortality of gopher frog, chorus frog and southern toad larvae among exposure treatments (F =1.78, 2df, P =0.35). Community-level mortality was 62%, 68%, and 62% when gopher frog, chorus frog, and southern toad larvae were directly exposed to ranavirus, respectively (Figure 5.2). While, directly exposed gopher frog, chorus frog, and southern toad larvae experienced 100%, 78%, and 76% mortality, respectively (Figure 5.2). Directly exposed gopher frog larvae transmitted ranavirus and caused 52% and 34% mortality in chorus frog and southern toad larvae, respectively (Figure 5.2 A). Directly exposed chorus frog larvae transmitted ranavirus and caused 70% and 58% mortality in gopher frog and southern toad larvae, respectively (Figure 5.2 B). Directly exposed southern toad larvae transmitted ranavirus and caused 80% and 32% mortality in gopher frog and chorus frog larvae, respectively (Figure 5.2 C). In the treatment when all individuals were directly exposed, mortality was 100%, 80% and 90% for gopher frog, chorus frog, and southern toad larvae, respectively. All individuals that died tested positive for ranavirus infection, and there was no mortality in the control treatment.

DISCUSSION

My results demonstrate that composition of a larval amphibian community and which species is exposed first to ranavirus will affect the outcomes of an outbreak. High community-

level mortality was observed in the Coastal Plains community regardless of which species was initially exposed to the virus. However, mortality was low in the Appalachian community if spotted salamander larvae were exposed first; whereas, mortality was high if wood frog larvae were initially exposed. Differences in outcomes may be related to species-specific susceptibilities or life history characteristics.

The Appalachian community was composed of species with low, moderate and high susceptibility according to single species challenges (Hoverman et al. 2011). The Coastal Plains community was composed of two moderately susceptible and a highly susceptible species (Hoverman et al. 2011, Chapter II). Highly susceptible species may exhibit greater virion shedding, which is supported by high viral loads in the tissue (>100,000 viral copies per 0.25 μg of gDNA) of morbid wood frog and gopher frog tadpoles (Gray and Miller, unpubl. data). In comparison, morbid spotted salamander larvae averaged about 7000 viral copies/0.25 μg, while morbid southern toads were infected at 45,000 viral copies/0.25 μg (Gray and Miller, unpubl. data). Highly infectious hosts are known to result in greater pathogen transmission than low susceptible hosts (Paull et al. 2012). Thus, a greater number of moderately susceptible hosts in the Coastal Plains community may have resulted greater viral loads in the aquatic mesocosms. Higher concentrations of ranavirus in water can lead to increased mortality (Pearman et al. 2004, Brunner et al. 2005).

Differences in community-level mortality may also have been influenced by the life history of the host species. The Appalachian community was composed of two anurans and a salamander species. Salamander larvae are sit-and-weight predators, while anuran larvae forage nearly continuously while swimming (Holomuzki 1989, Wyman 1998, Altig and Taylor 2003, Altig et al. 2007) thereby facilitating contact with other individuals. Thus, the Coastal Plains

community may have experienced greater transmission of ranavirus as a result of anuran hosts moving and contacting each other more. Further, anuran larvae are scavengers and known to eat dead conspecifics; whereas, salamander larvae primarily consume micro-invertebrates (e.g., zooplankton, Holomuzki, 1989). It is known that necrophagy is an efficient transmission route for ranavirus (Gray et al. 2009), and that ingestion of ranavirus can result in faster mortality than exposure to virions in a water bath (Hoverman et al. 2010). Thus, greater levels of mortality in the Coastal Plains community may have been a consequence of a greater number of anuran scavengers.

Directly exposed wood frogs caused 42% and 12% mortality in naïve chorus frog and spotted salamander larvae, respectively. This level of mortality was nearly identical for chorus frog tadpoles when this species was directly exposed to ranavirus inoculum. Thus, infected wood frog tadpoles are able to transmit ranavirus to chorus frog tadpoles very effectively. Moreover, the amount of mortality observed in spotted salamander larvae was 2X when this species was directly exposed to ranavirus inoculum. Thus, wood frogs might be considered an amplification species considering their high mortality during exposure, and efficient transmission of ranavirus to other species (Paull et al. 2012).

Directly exposed chorus frog tadpoles efficiently transmitted ranavirus to wood frog tadpoles resulting in 72% mortality, but transmission to spotted salamander larvae was minimal (4%). Differences in transmission may be related to the higher susceptibility of wood frogs to ranavirus compared to spotted salamander larvae (Hoverman et al. 2011), or perhaps wood frog and chorus frog tadpoles occupied similar microhabitats in the mesocosms increasing their contact rate. These results suggest that infected chorus frog tadpoles could initiate an outbreak in

a population of wood frog tadpoles, and may be a good reservoir species when highly susceptible species are not present.

Spotted salamander larvae had low susceptibility to ranavirus and were inefficient in transmitting the pathogen to other species. Spotted salamanders are frequently reported in ranavirus die-offs (Green et al. 2002, Greer et al. 2005, Gahl and Calhoun 2010). Given the low mortality demonstrated during my study as well as in laboratory experiments (Hoverman et al. 2011), it is unlikely that spotted salamander larvae would initiate an outbreak of ranavirus. I hypothesize that mortality of spotted salamander larvae observed in the wild is a consequence of high viral loads in the aquatic environment due to shedding by highly susceptible species or presence of a stressor that reduces immune function.

In the Coastal Plain community, all species efficiently transmitted ranavirus resulting in significant mortality of other species. These results suggest that communities composed of moderate and highly susceptible species are at risk of ranaviral disease emergence. Using results from Hoverman et al. (2011) and Chapter II, species with single challenge mortality rates >40% could be considered high-risk species capable of initiating an outbreak. Although this recommendation remains to be tested, natural resource practitioners might use single-challenge results to identify possible hotspots for ranavirus emergence by considering species composition. From my results, if a community contains at least three moderate to highly susceptible species, community-level mortality could be high (e.g., >60%) when ranavirus is present in the aquatic environment.

Mortality observed during my mesocosm experiments was nearly identical to single species challenges in the laboratory for the Appalachian community (Hoverman et al. 2011,

Chapter II); however, it was elevated for two species (chorus frog and southern toad) in the Coastal Plains community. Single challenge experiments were performed in isolation (Hoverman et al. 2011); thus, contact with other individuals was prevented. Also, the viral load in single challenge experiments was 10^3 PFU/mL, which may be exceeded during ranavirus die-offs (Rojas et al. 2005). I hypothesize that chorus frog and southern toad mortality may have been elevated in the Coastal Plains community as a result of greater contact rates and viral load than in the single challenge experiments, and that these mechanisms were greater than in the Coastal Plains community compared to the Appalachian community. Water quality and temperature were similar during both experiments (Brenes, unpubl. data), and did not exceed levels known to negatively affect amphibian larvae (Nelms et al. 2012, Gray and Miller 2013). Given these results, single challenge studies (e.g., Hoverman et al. 2011, Chapter II) might be considered as best-case scenarios for mortality in wild populations.

According to Paull et al. (2012), environments that contain highly infectious species can be consider diseases hotspots, which are sites or regions with particularly high levels of pathogen prevalence (percentage of infected host), pathogen intensity (pathogens per infected host), or transmission rates. These areas can serve as "source areas" from which the pathogen can disperse to less infected areas across a landscape. Gray and Miller (2013) proposed that areas with ranavirus prevalence exceeding 40% should be considered hotspots, because ranavirus infection and mortality are strongly correlated in many amphibian species. Based on these definitions, when wood frogs or chorus frogs in the Appalachian community were exposed to ranavirus or any species of the Coastal Plain community was exposed, a disease hotspot existed. My semi-natural mesocosms are not as complex as most amphibian breeding sites; thus, direct inferences to natural populations should be made cautiously. Nonetheless, they provide a basis for testing

future hypotheses on community-level transmission dynamics of ranaviruses, and evidence that community composition can affect the likelihood of a ranavirus outbreak. Results from my mesocosm experiments may represent a best-case scenario for amphibian survival, because a pathogen was the only known stressor that was present.

Overall, my experiments demonstrate that amphibian communities have heterogeneous degrees of susceptibility and capacity of transmission of ranavirus, and that these species specific differences can have a direct impact on the role that different species play in the persistence of the pathogen in the environment (Haydon et al. 2002, Woolhouse et al. 2005, Beldomenico and Begon 2010b, a, Paull et al. 2012). The presence of highly susceptible species with high capacities of transmission can create diseases hotspots and sources areas that will promote the persistence and dispersion of the pathogen across the environment, resulting in recurrent mortality events that could diminish populations and entire amphibian communities. The establishment of broad scale surveillance efforts to identify the location of hotspots and sources areas as well as research to determine the level of susceptibility of species that could function as superspreading species like the ones identified in these experiments, is of great importance in the creation of amphibian conservation strategies that would take into consideration the landscape structure linking these areas of high pathogen prevalence, with spatiotemporal metacommunity processes like species dispersal and community structure. The identification of highly susceptible species capable to cause outbreaks, could be used as predictors for forecasting amphibian populations outbreaks, helping to determine the risk factor of particular environments, guiding conservation efforts to determinate high risk areas. These conservationist strategies would help us understand the factors driving ranavirus outbreaks, recurrent die-off events, and most importantly help us to identify high risk areas where outbreaks are likely to occur.

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APPENDICES

APPENDIX A

CHAPTER II: LIFE HISTORY MATTERS: HOST CHARACTERISTICS AFFECT SUSCEPTIBILITY TO AN EMERGING PATHOGEN

TABLES AND FIGURES

Table 2.1. Species tested for ranavirus susceptibility via water bath. Table contain species tested by Hoverman et al. (2011) and the species tested during the 2011 and 2012 experiments.

Family	Acronym	Common name	Scientific name	Tested
Ranidae	LISY	Wood frog	*Lithobates sylvaticus*	Hoverman et al. 2011
	LICAP	Gopher frog	*Lithobates capito*	Hoverman et al. 2011
	LISP	Southern leopard frog	*Lithobates sphenocephala*	Hoverman et al. 2011
	LIPI	Northern leopard frog	*Lithobates pipiens*	Hoverman et al. 2011
	LIPA	Pickerel frog	*Lithobates palustris*	Hoverman et al. 2011
	LICL	Green frog	*Lithobates clamitans*	Hoverman et al. 2011
	LICA	American bullfrog	*Lithobates catesbeiana*	Hoverman et al. 2011
	LIAR	Crawfish frog	*Lithobates areolata*	2011
	LITE	Common frog	*Rana temporaria*	2012
	LIBO	Foothill Yellow-legged Frog	*Lithobates boylii*	2011
Hylidae	HYCH	Cope's gray tree frog	*Hyla chrysoscelis*	Hoverman et al. 2011
	PSFE	Upland chorus frog	*Pseudacris feriarum*	Hoverman et al. 2011
	PSTR	Western chorus frog	*Pseudacris triseriata*	Hoverman et al. 2011
	PSBR	Mountain chorus frog	*Pseudacris brachyphona*	Hoverman et al. 2011
	PSOR	Ornate chorus frog	*Pseudacris ornata*	2012
	PSRE	Pacific treefrog	*Pseudacris regilla*	2011
Bufonidae	ANAM	American toad	*Anaxyrus americanus*	Hoverman et al. 2011
	ANTE	Southern Toad	*Anaxyrus terrestris*	2011
	ANBO	Western Toad	*Anaxyrus boreas*	2011
Microhylidae	GACA	Eastern narrow-mouthed toad	*Gastrophryne carolinensis*	Hoverman et al. 2011
Scaphiopodidae	SCHO	Eastern spadefoot	*Scaphiopus holbrookii*	Hoverman et al. 2011
	SCCO	Couch's spadefoot	*Scaphiopus couchi*	2012
Pipidae	XELA	Africa clawed frog	*Xenopus laevis*	2012
Ambystomatidae	AMTI	Tiger salamander	*Ambystoma tigrinum*	Hoverman et al. 2011
	AMOP	Marbled salamander	*Ambystoma opacum*	Hoverman et al. 2011
	AMMA	Spotted salamander	*Ambystoma maculatum*	Hoverman et al. 2011
	AMTA	Mole salamander	*Ambystoma talpoideum*	Hoverman et al. 2011
	AMBA	Streamside Salamander	*Ambystoma barbouri*	2011
	AMTE	Smallmouth salamander	*Ambystoma texanum*	2012
	AMGR	Northwestern salamander	*Ambystoma gracile*	2012
	AMJE	Jefferson salamander	*Ambystoma jeffersonianum*	2012
Salamandridae	NOVI	Red-spotted newt	*Notophthalmus viridescens*	Hoverman et al. 2011
	NOPE	Striped Newt	*Notophthalmus perstriatus*	2011
	CYPY	Japanese Fire Newt	*Cynops pyrrhogaster*	2011
Plethodontidae	HESC	Four toed Salamander	*Hemidactylium scutatum*	2011

Table 2.2. Egg mass collection sites and number of egg masses collected for each of the 16 amphibian species tested in the 2011 and 2012 experiments.

Common name	Scientific name	State	County	Location	lat-long	Egg masses
Crawfish frog	*Lithobates areolata*	KY	McCracken	Paducah	37.08374° N, 88.45465° W	6
Common frog	*Rana temporaria*	UK	NA	Scotland	55.98253° N, 004.00592° W	2
Foothill Yellow-legged Frog	*Lithobates boylii*	CA	Trinity	South Fork Trinity River	40.88986° N, 123.60237° W	1
Ornate chorus frog	*Pseudacris ornata*	AL	Taylor	Taylor, GA	32.57855° N, 84.26949° W	2
Pacific treefrog	*Pseudacris regilla*	CA	San Luis Obispo	San Luis Obispo	35.2828° N, 120.6586° W	2
Southern Toad	*Anaxyrus terrestris*	MS	Forrest	Fountain Blue Apts	31.3342° N, 89.34026°W	1
Western Toad	*Anaxyrus boreas*	CA	Trinity	South Fork Trinity River	40.88986° N, 123.60237° W	4
Couch's spadefoot	*Scaphiopus couchi*	AZ	Pima	Sabino Canyon	32.3167° N,110.8167 ° W	2
Africa clawed frog	*Xenopus laevis*	NA	NA	Retailer	NA	1
Streamside Salamander	*Ambystoma barbouri*	KY	Franklin	Frankfort	38.2018° N,84.8736 ° W	4
Smallmouth salamander	*Ambystoma texanum*	KY	McCracken	Paducah	37.08374° N, 88.45465° W	5
Northwestern salamander	*Ambystoma gracile*	CA	Trinity	South Fork Trinity River	40.88986° N, 123.60237° W	3
Jefferson salamander	*Ambystoma jeffersonianum*	KY	Franklin	Frankfort	38.3334° N,84.59102 ° W	4
Striped Newt*	*Notophthalmus perstriatus*	NE	Douglas	Omaha zoo	NA	6
Japanese Fire Newt	*Cynops pyrrhogaster*	NA	NA	Private	NA	1
Four toed Salamander	*Hemidactylium scutatum*	KY	Adair	Neatville	37.1151° N, 85.12545° W	6

Table 2.3. Species-level characteristics for the species used in the experiments, the table includes the 19 species tested by Hoverman et al. (2011) as well as the 16 species tested during the 2011 – 2012 experiments. Data were gathered from multiple literature sources (Altig 1970, Conant and Collins 1998, Petranka 1998, McDiarmid and Altig 1999, Savage 2002, Altig and McDiarmid 2007, Wells 2007). Data includes, species tested by Hoverman et al. (2011) and species tested in 2011 and 2012. For species names see table 2.1.

Species	Breeding habitat	Breeding time	Time to metamorphosis (days)	Size at metamorphosis (mm)	Clutch size (# of eggs)	Hatch time (days)	Adult size (mm)	Maturity (years)	Range size (Km^2)	Longevity (years)	Aquatic index	Length of Reproduction (mm)	Egg size	Gregarious
AMGR	3.00	2.00	390.00	60.50	150.00	40.00	72.50	2.00	104614	5.00	3.00	90.00	3	0
AMMA	2.50	2.00	203.50	51.00	625.00	34.00	154.50	2.50	3557209	23.50	4.00	60.00	2	0
AMOP	2.00	1.00	150.00	57.50	118.50	90.00	98.50	2.00	1637972	9.00	4.00	90.00	3	0
AMTA	2.00	1.00	105.00	49.00	445.00	33.00	87.50	2.00	546868	8.00	4.00	60.00	2	0
AMTI	2.00	3.00	100.00	62.00	3700.00	34.50	195.00	2.00	1857253	16.00	4.00	120.00	3	0
AMTE	1.00	2.00	80.00	53.00	8.50	35.00	83.00	2.00	550780	12.30	4.00	45.00	3	1
AMBA	4.00	3.00	52.50	39.00	260.00	17.50	67.00	2.00	18228	12.30	4.00	77.50	3	0
AMJE	3.00	3.00	90.00	65.00	210.00	59.50	70.00	2.50	172000	3.00	4.00	90.00	3	0
ANAM	2.00	3.00	57.50	9.50	7000.00	7.50	70.50	3.00	4928210	4.50	2.00	90.00	1	1
ANBO	2.00	2.50	37.50	13.00	5200.00	6.50	90.50	5.00	723420	7.50	2.00	60.00	1	1
ANTE	2.00	2.50	32.50	8.75	3250.00	3.00	67.00	2.00	255996	10.00	3.00	240.00	1	1
GACA	1.50	4.00	45.00	10.25	775.00	1.25	27.00	1.50	1516976	4.00	4.00	180.00	1	0
HYCH	3.00	4.00	55.00	16.50	90.00	5.00	41.50	2.00	2484281	7.90	3.00	60.00	2	0
PSBR	1.50	2.00	47.00	12.00	157.50	8.50	28.50	1.00	255456	2.85	3.00	60.00	1	1
PSFE	2.50	2.00	55.00	10.00	1000.00	8.50	27.00	1.00	868550	2.85	3.00	60.00	1	1
PSTR	2.50	2.00	55.00	10.00	1000.00	8.50	29.00	1.00	698650	2.85	3.00	65.00	1	1
PSOR	1.00	2.00	90.00	15.00	55.00	7.00	35.00	2.00	124206	3.50	4.00	120.00	2	0
PSRE	2.00	2.50	67.50	13.50	49.00	6.00	36.50	2.00	472335	2.85	3.00	180.00	3	0
NOVI	3.50	4.00	90.00	20.00	350.00	27.50	89.50	1.35	2373532	10.50	1.00	120.00	2	0
NOPE	4.00	1.50	139.00	18.00	3.50	27.50	28.50	1.35	2373532	12.00	1.00	120.00	2	0
CYPY	4.00	3.50	120.00	25.00	200.00	35.00	115.00	2.50	145882	15.00	1.00	60.00	2	0
LICAP	2.50	2.00	161.50	32.50	1450.00	8.50	77.00	1.75	146967	6.50	4.00	90.00	1	1
LICA	4.00	3.00	450.00	60.00	20000.00	8.00	121.00	2.00	4432306	7.50	2.00	100.00	1	1
LICL	3.00	4.00	242.50	29.50	3000.00	8.00	73.50	1.00	3541109	6.50	2.00	80.00	1	1
LIPA	3.00	2.00	75.00	23.00	3000.00	14.50	59.50	2.00	3348573	6.50	3.00	150.00	1	1
LIPI	3.50	2.00	100.00	34.00	3800.00	14.50	70.50	2.00	6852031	9.00	2.00	90.00	1	1
LISP	3.00	3.00	62.50	26.50	1350.00	14.50	70.50	2.00	1649839	6.50	2.00	90.00	1	1
LISY	1.50	2.00	65.00	17.00	3000.00	12.00	52.50	2.00	8884571	4.00	3.00	60.00	1	1
LIAR	1.00	2.00	90.00	30.00	5000.00	10.00	82.80	3.50	234444	5.00	3.00	38.50	1	1
LIBO	4.00	2.50	150.00	15.50	900.00	21.00	65.00	0.50	127850	6.50	2.00	65.00	2	0
LITE	1.00	2.00	60.00	18.00	4000.00	10.00	50.00	3.00	2358000	7.00	3.00	90.00	1	1
SCHO	1.00	4.00	37.00	10.25	4200.00	3.00	50.50	1.50	1003376	9.00	4.00	120.00	1	1
SCCO	1.00	4.00	12.00	11.00	3000.00	1.00	66.00	2.50	525138	12.00	4.00	120.00	1	1
HESC	3.00	4.00	30.00	50.00	24.00	25.00	67.00	2.30	389048	5.50	4.00	30.00	3	0
XELA	4.00	3.00	77.00	15.00	1500.00	2.50	65.00	0.80	481000	15.50	1.00	65.00	1	0

Table 2.4. Best fitted models describing the relationship between infections to the FV3 isolate ranavirus and natural history traits. Smallest Akaike's information criterion (AIC) represents the best model explaining the relation between infection and natural history traits ($\Delta i \geq 2$). Models with the lowest AIC are ranked first. R^2=stepwise multiple regression value, wi=model weight. Numbers in parenthesis represent ordinal variables ranks (see text).

Best models with lowest AIC	**Variables**	**R^2**	**RMSE**	**AICc**	**Δ_i**	**w_i**
Hatching time, Aquatic index	2	0.1821	30.9856	345.8693	0	0.057764
Clutch size, Hatching time	2	0.1752	31.1168	346.165	0.2957	0.0498249
Clutch size, Hatching time, Egg size	3	0.2347	30.4524	346.2785	0.4092	0.0470761
Clutch size, Hatching time, Aquatic index	3	0.2325	30.4969	346.3808	0.5115	0.0447287
Breeding time, Aquatic index	2	0.1655	31.2982	346.5719	0.7026	0.0406527
Hatching time, Egg size	2	0.1596	31.409	346.8192	0.9499	0.0359243
Aquatic index	1	0.0954	32.0895	346.8374	0.9681	0.0355989
Hatching time, Aquatic index (3), Egg size (1)	3	0.2216	30.7122	346.8732	1.0039	0.0349674
Hatching time, Aquatic index (3),Aquatic index (1)	3	0.2139	30.8634	347.217	1.3477	0.0294447
Hatching time, Aquatic index (3), Egg size (2)	3	0.2135	30.8712	347.2347	1.3654	0.0291853
Hatching time	1	0.0753	32.4444	347.6075	1.7382	0.0242221
Breeding time (2),Clutch size, Hatching time	3	0.2041	31.0561	347.6527	1.7834	0.0236808
Size to metamorphosis, Aquatic index (3)	2	0.1386	31.7999	347.6851	1.8158	0.0233003
Breeding habitat (2),Hatching time, Aquatic index (3)	3	0.2026	31.084	347.7155	1.8462	0.0229488
Hatching time, Aquatic index (3)	2	0.1359	31.8497	347.7946	1.9253	0.0220589
Breeding habitat (2), Hatching time, Aquatic index (3), Egg size (1)	4	0.2644	30.3484	347.8225	1.9532	0.0217533

Table 2.5. Best fitted models describing the relationship between infection to the Ranaculture (RC) isolate ranavirus and natural history traits. Smallest Akaike's information criterion (AIC) represents the best model explaining the relation between infection and natural history traits ($\Delta i \geq 2$). Models with the lowest AIC are ranked first. R^2=stepwise multiple regression value, wi=model weight. Numbers in parenthesis represent ordinal variables ranks (see text).

Best models with lowest AIC	**Variables**	**R^2**	**RMSE**	**AICc**	**Δ_i**	**w_i**
Breeding habitat (3), Breeding time (2)	2	0.2161	33.1775	350.6536	0	0.097098
Breeding habitat (3), Breeding time (1), Breeding time (2)	3	0.2364	33.2679	352.4685	1.8149	0.0391841
Breeding habitat (3), Breeding time (2), clutch size	3	0.2326	33.3521	352.6455	1.9919	0.0358653

Table 2.6. Best fitted models describing the relationship between infection to the Smoky mountain (SM) isolate ranavirus and natural history traits. Smallest Akaike's information criterion (AIC) represents the best model explaining the relation between infection and natural history traits ($\Delta i \geq 2$). Models with the lowest AIC are ranked first. R^2=stepwise multiple regression value, wi=model weight. Numbers in parenthesis represent ordinal variables ranks (see text).

Best models with lowest AIC	**Variables**	**R^2**	**RMSE**	**AICc**	**Δ_i**	**w_i**
Hatch time, Longevity, Aquatic index (1), Distance	4	0.9032	14.5969	126.2818	0.0000	0.5623
Hatch time, Aquatic index (1), Distance	3	0.7933	20.1085	128.7131	2.4313	0.1667

Table 2.7. Natural history traits influencing susceptibility of the FV3 isolate presenting the higher relative weights during whole model averaging. Weight=overall weight of variable in the global model, MA STβ= Model average standardized beta value, MA-SE =Model average standard error. Model averaging was conducted according to Anderson (2008) and included all possible model combinations. Aquatic index: 1= low dependence to water (i.e. only found in water during reproduction), 2 = medium dependence to water (i.e. usually found near water), and 3 = complete dependence to water (i.e. fully aquatic); breeding habitat: 1= temporary ponds, 2=semi-permanent ponds, and 3= permanent bodies of water; breeding time: 1= early spring, 2= late spring, and 3= summer; egg size: 1= small, 2= large. See text for variable units.

Variable	Weight	MA STβ	MA-SE
Hatching time	5.4130	-0.1797	0.0063
Aquatic index (2)	3.9886	0.1372	0.0062
Aquatic index (3)	2.3815	-0.0803	0.0038
Clutch size	1.3948	0.0439	0.0048
Egg size (2)	1.1855	-0.0457	0.0042
Breeding time (3)	1.0058	-0.0265	0.003
Aquatic index (3)	1.005	0.0352	0.0023
Breeding time (2)	0.6629	0.0177	0.0021
Breeding time (1)	0.6269	0.019	0.0029
Breeding habitat (3)	0.3827	-0.0081	0.0014
Breeding habitat (1)	0.3198	-0.0067	0.0011
Size to metamorphosis	0.2197	-0.0051	0.0013
Time to metamorphosis	0.2024	-0.004	0.001
Breeding habitat (2)	0.1401	0.003	0.0006
Egg size (1)	0.1156	0.0079	0.0012

Table 2.8. Natural history traits influencing susceptibility of the ranaculture isolate (RC) presenting the higher relative weights during whole model averaging. Weight=overall weight of variable in the global model, MA STβ= Model average standardized beta value, MA-SE =Model average standard error. Model averaging was conducted according to Anderson (2008) and included all possible model combinations. Aquatic index: 1= low dependence to water (i.e. only found in water during reproduction), 2 = medium dependence to water (i.e. usually found near water), and 3 = complete dependence to water (i.e. fully aquatic); breeding habitat: 1= temporary ponds, 2=semi-permanent ponds, and 3= permanent bodies of water; breeding time: 1= early spring, 2= late spring, and 3= summer; egg size: 1= small, 2= large. See text for variable units.

Variable	Weight	MA STβ	MA-SE
Breeding habitat (3)	5.5489	-0.1726	0.0045
Breeding time (2)	4.8632	0.1262	0.004
Breeding habitat (2)	3.9614	0.116	0.0032
Breeding time (3)	3.6039	-0.0939	0.003
Breeding time (1)	2.4655	0.0741	0.0026
Breeding habitat (1)	2.2639	-0.0765	0.0045
Clutch size	1.2031	-0.0203	0.0024
Aquatic index (2)	1.1253	0.0206	0.0025
Range size	1.1088	0.0176	0.0024
Aquatic index (1)	0.7458	-0.0134	0.0016
Aquatic index (3)	0.2379	-0.0035	0.0011
Maturity	0.1912	-0.0069	0.0018
Hatching time	0.1827	-0.0029	0.0008
Egg size (2)	0.1642	0.0019	0.0006
Egg size (1)	0.1346	-0.0015	0.0005
Time to reproduction	0.1172	0.0032	0.0008

Table 2.9. Natural history traits influencing susceptibility of the Smoky mountain isolate (SM) presenting the higher relative weights during whole model averaging. Weight=overall weight of variable in the global model, MA STβ= Model average standardized beta value, MA-SE =Model average standard error. Model averaging was conducted according to Anderson (2008) and included all possible model combinations. Aquatic index: 1= low dependence to water (i.e. only found in water during reproduction), 2 = medium dependence to water (i.e. usually found near water), and 3 = complete dependence to water (i.e. fully aquatic); breeding habitat: 1= temporary ponds, 2=semi-permanent ponds, and 3= permanent bodies of water; breeding time: 1= early spring, 2= late spring, and 3= summer; egg size: 1= small, 2= large. See text for variable units.

Variable	Weight	MA STβ	MA-SE
Aquatic index (1)	12.9543	0.8713	0.0066
Hatching time	11.6798	-0.7548	0.0052
Aquatic index (3)	11.0233	-0.7357	0.0115
Distance	5.5019	0.3957	0.0038
Longevity	3.0172	-0.2371	0.0155
Breeding habitat (3)	0.7988	-0.0105	0.002
Breeding habitat (2)	0.5903	0.0078	0.0015
Adult size	0.5052	0.0105	0.0029
Maturity	0.4757	0.0122	0.0029
Breeding habitat (1)	0.4559	-0.006	0.0011
Aquatic index (2)	0.3904	0.0307	0.0041
Clutch size	0.2039	0.0037	0.001
Breeding time (3)	0.1152	0.0007	0.0002
Range size	0.1129	0.0052	0.0015
Time to reproduction	0.0920	-0.0005	0.0001
Breeding time (2)	0.0852	0.0005	0.0001

Figure 2.1. Phylogenetic hypothesis among the 35 amphibian species included in the study. Branch lengths are presented as divergence times (millions of years). When divergence times were not available for a set of taxa, the branch length was divided equally among the taxa.

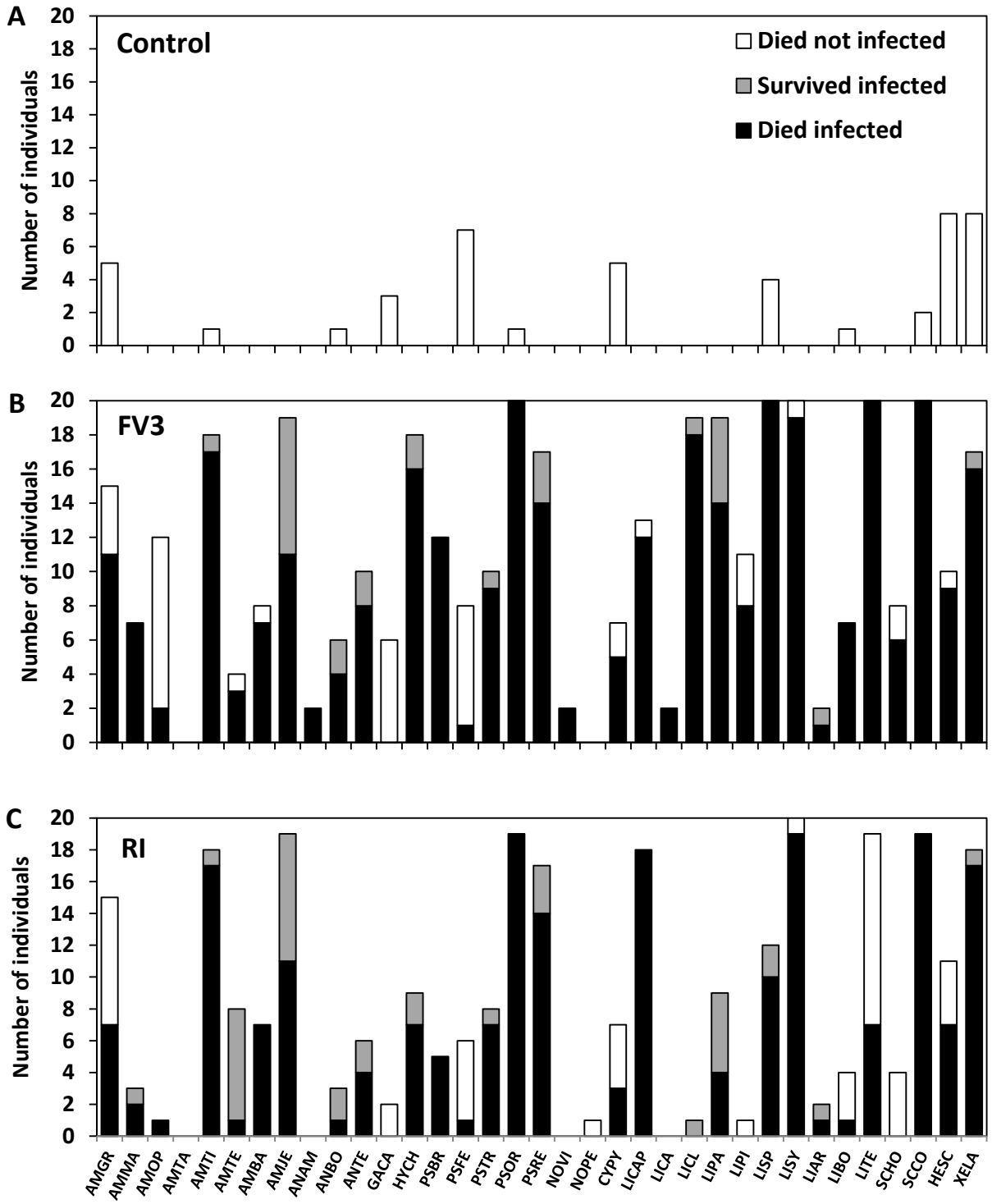

Figure 2.2. Infection and mortality for 35 larval amphibian species exposed to A= the virus free control, B=frog virus 3 (FV3), or C= Ranaculture isolate (RI). The experiment lasted 21 days.

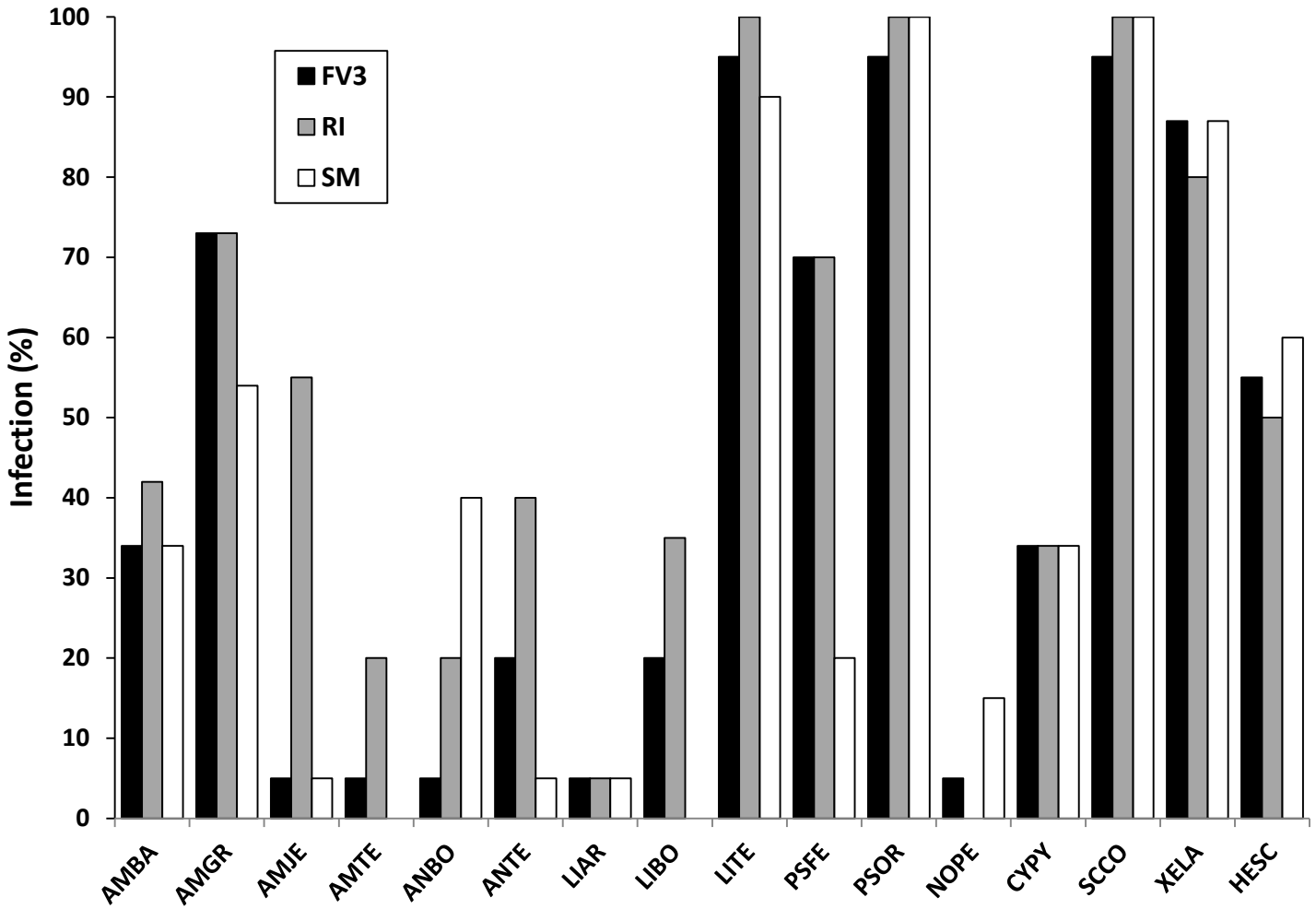

Figure 2.3. Percentage of mortality of the species tested to the three ranavirus isolates FV3= Frog virus 3, RI= Ranaculture isolate, and SM= Smoky mountain isolate. Data include 16 species tested during the 2011 and 2012 experiments. The experiment lasted 21 days.

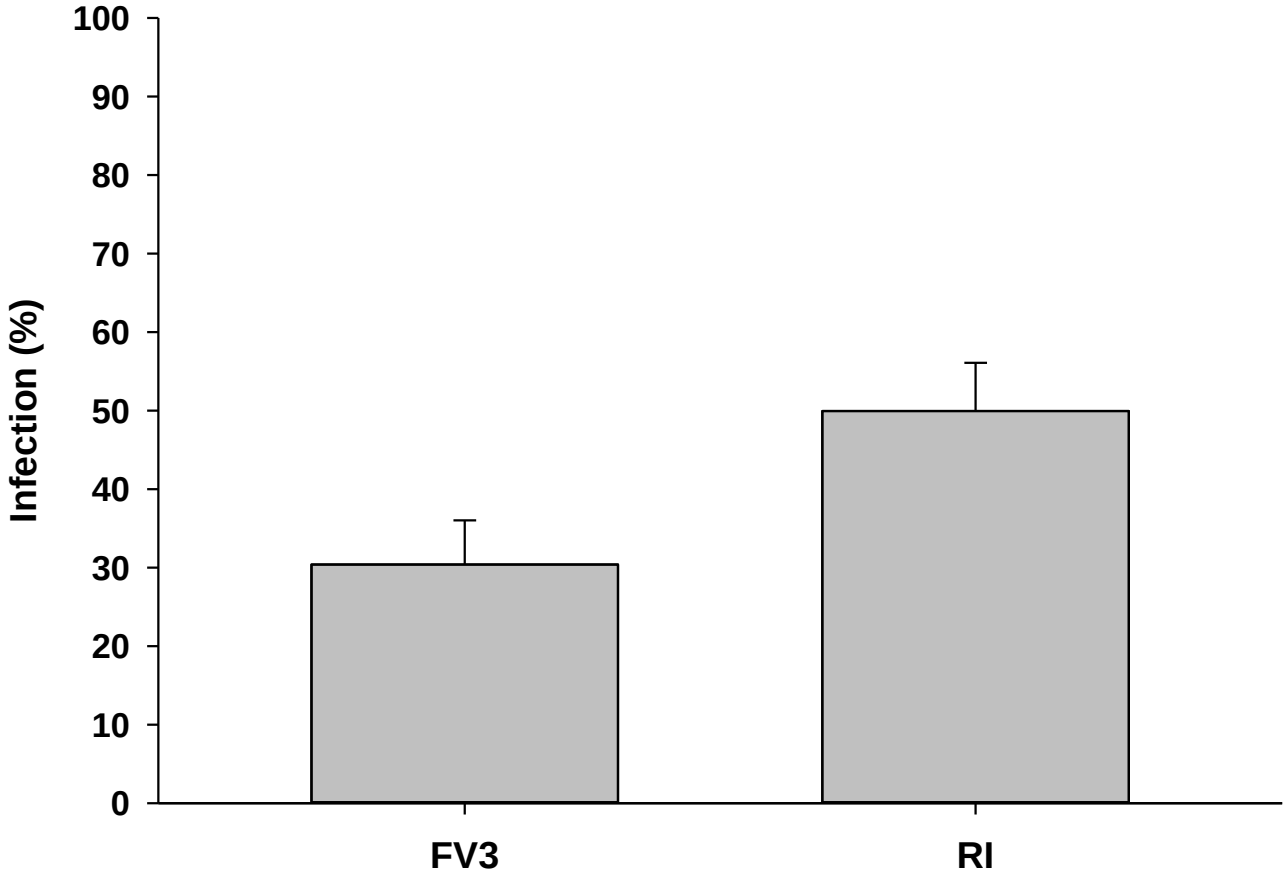

Figure 2.4. Percentage of infection of the species tested to the two ranavirus isolates FV3= Frog virus 3, and RC= Ranaculture isolate. Data include 35 species tested during the Hoverman et al. (2011) experiments and the 2011 and 2012 experiments. The experiment lasted 21 days.

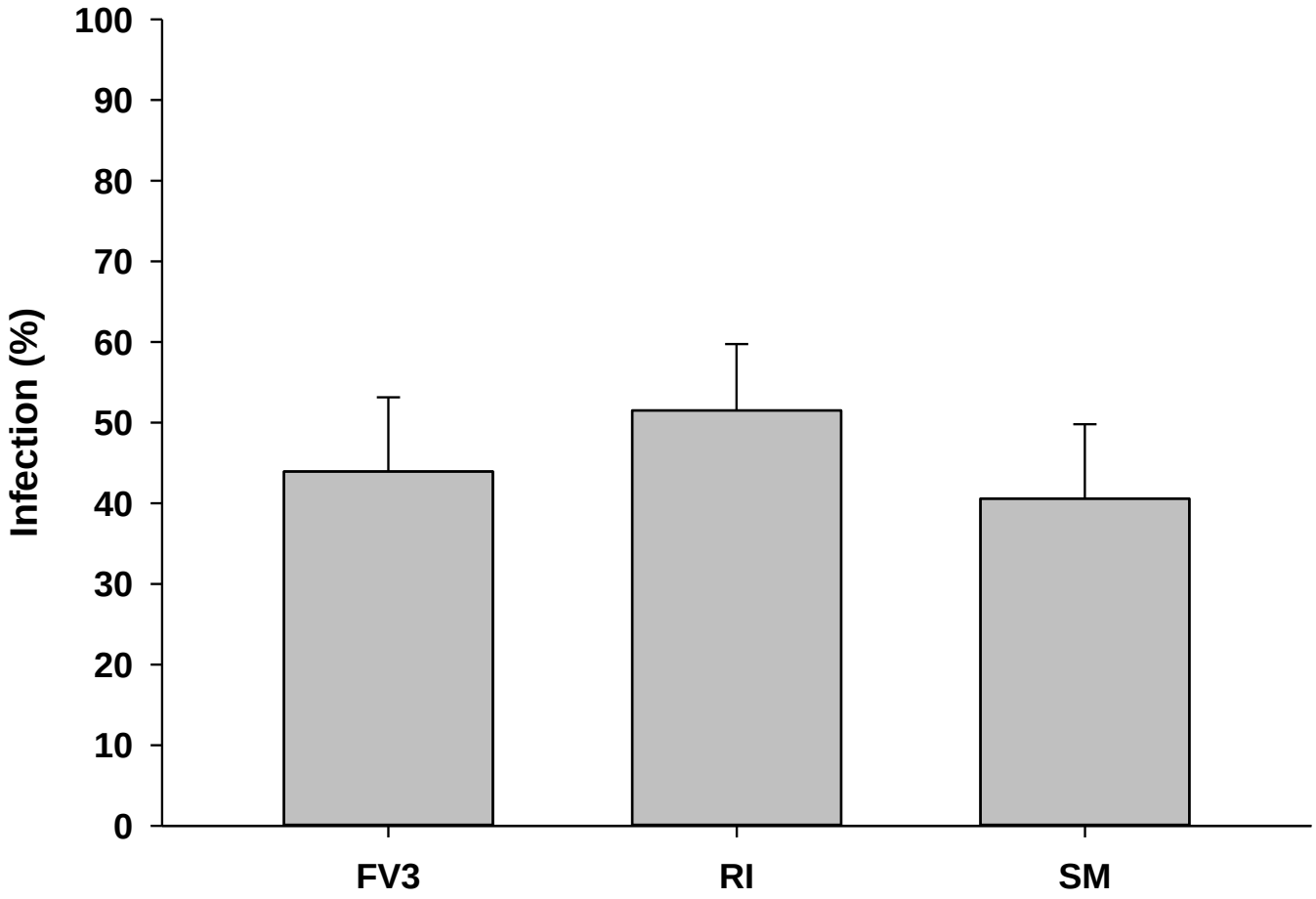

Figure 2.5. Percentage of infection of the species tested to the three ranavirus isolates FV3= Frog virus 3, RC= Ranaculture isolate, and SM= Smoky mountain isolate. Data include 16 species tested during the 2011 and 2012 experiments. The experiment lasted 21 days.

APPENDIX B

CHAPTER III: SUSCEPTIBILITY OF FIVE FISH AND THREE TURTLE SPECIES TO THREE DIFFERENT RANAVIRUS ISOLATES FROM DIFFERENT ECTOTHERMIC CLASSES

TABLES AND FIGURES

Table 3.1. Place of origin of the five fish and three turtle species used during the challenge experiments. All fish were reared from fry in independent outdoor concrete troughs, with no contact with other species, no heating, and constant water flow, mimicking natural conditions. Turtles were raised from eggs, each species independently.

Common Name	**Scientific name**	Origin
Tilapia	*Oreochromis niloticus niloticus*	Greenwater Fish Farm**,** Milan, TN
Channel catfish	*Ictalurus punctatus*	Greenwater Fish Farm**,** Milan, TN
Mosquito fish	*Gambusia affinis*	Alabama Aquarium & Pond Services, Inc. Birmingham, AL
Bluegill	*Lepomis macrochirus*	*Bell Springs* Fish *Hatchery.* Riceville TN
Fathead minnow	*Pimephales promelas*	*Bell Springs Fish Hatchery.* Riceville TN
Soft shell turtle	*Apalone ferox*	JP Pets. Sanford, FL
Eastern river Cooter	*Pseudemys concinna*	JP Pets. Sanford, FL
Mississippi Map Turtle	*Graptemys kohni*	*Backwater Reptiles.* Sacramento, CA

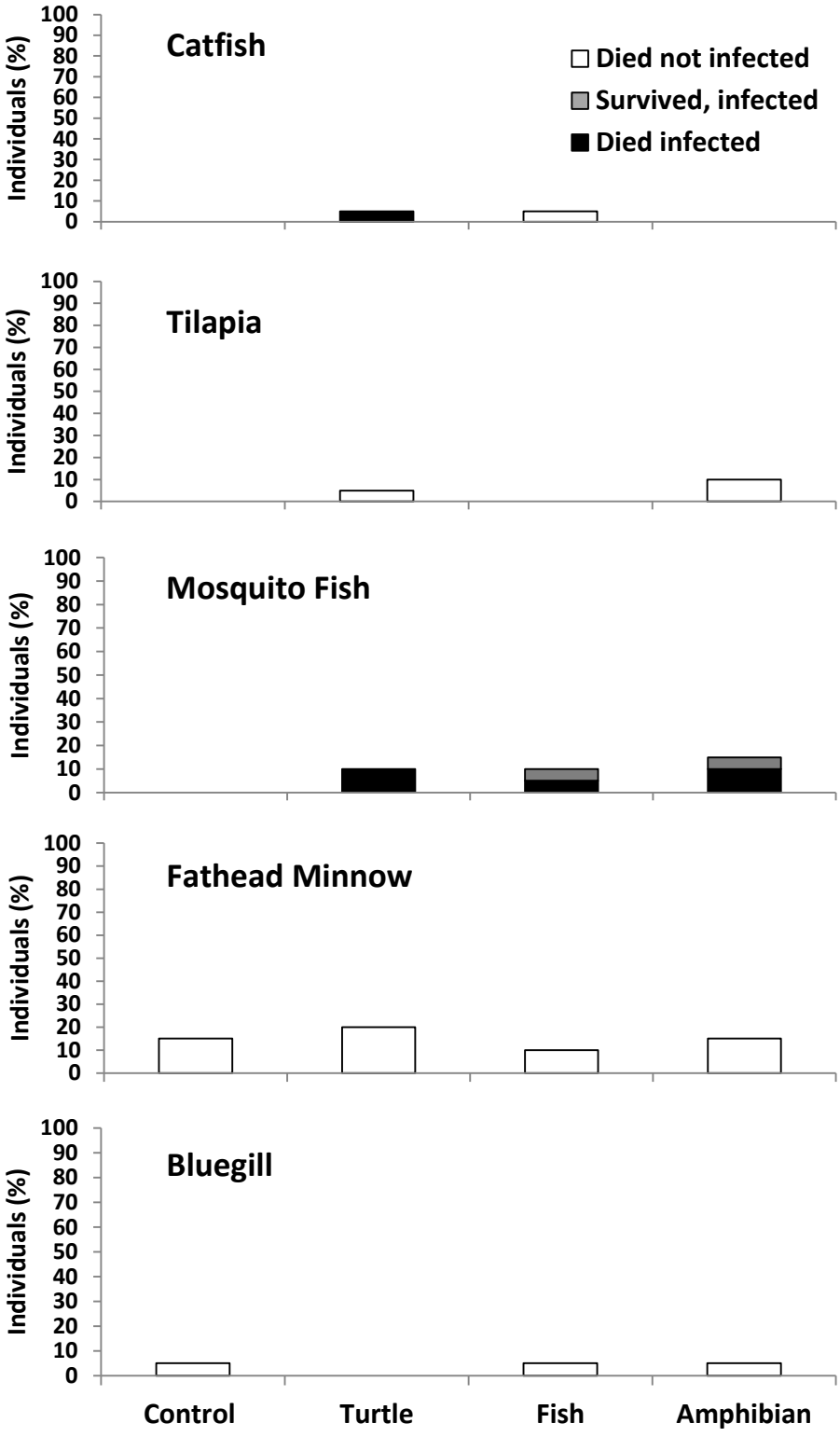

Figure 3.1. Percent mortality of five fish species exposed to three ranavirus isolates from different ectothermic hosts; turtle, fish, and amphibian. Results are based on exposure of 20 individuals for 28 days.

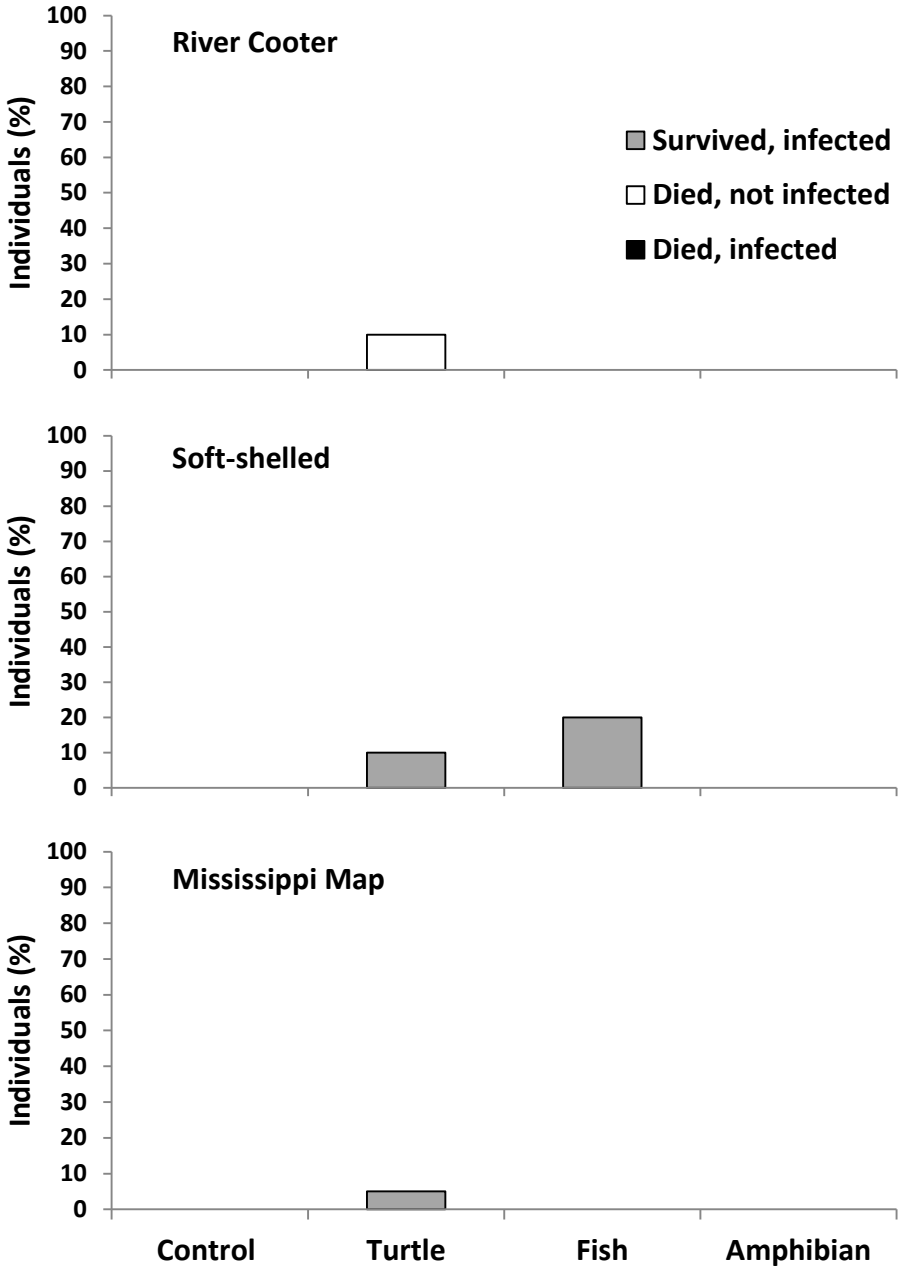

Figure 3.2. Percent mortality of turtles species exposed to three ranavirus isolates from different ectothermic hosts; turtle, fish, and amphibian. Results are based on exposure of 20 individuals for 28 days.

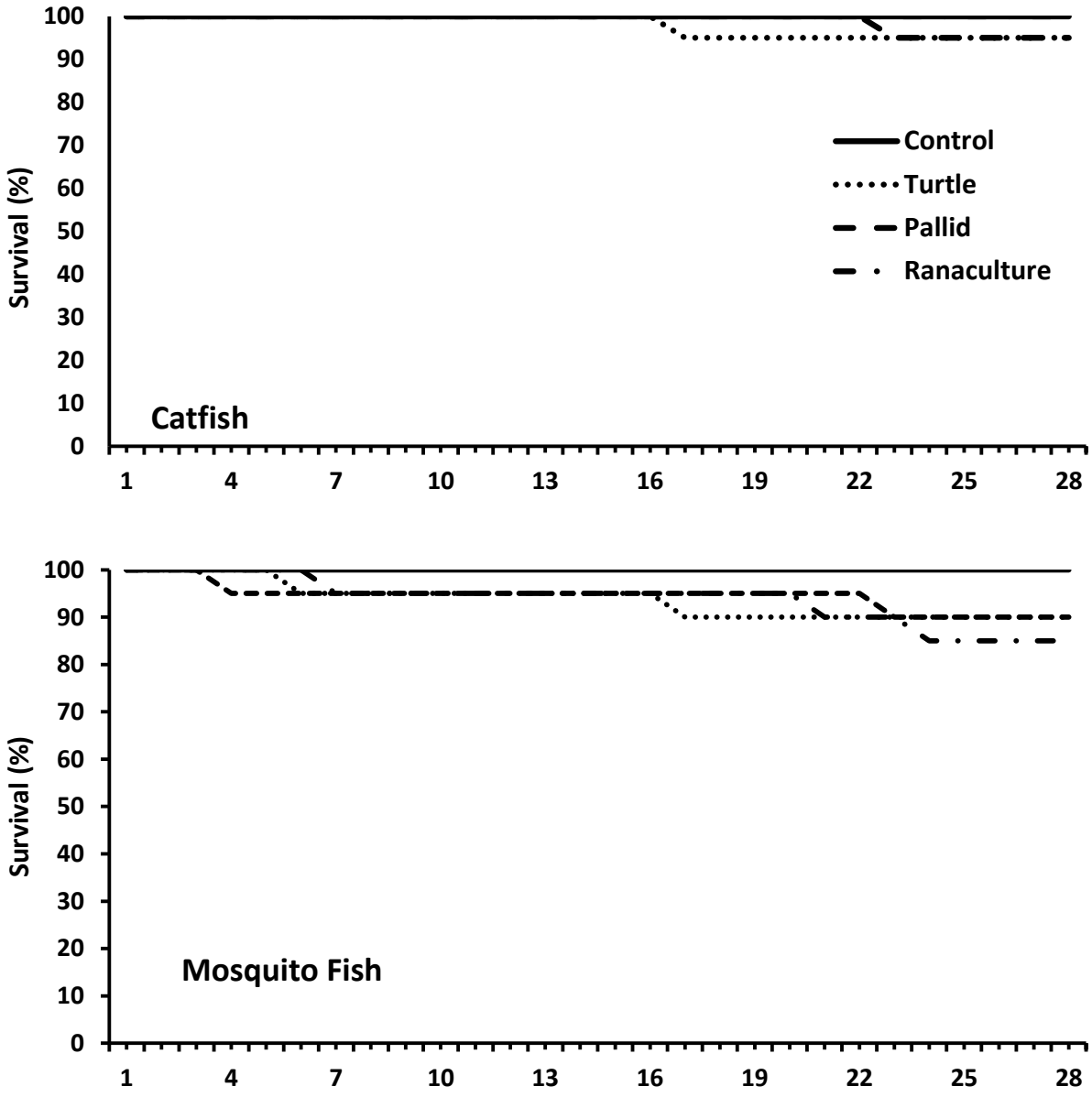

Figure 3.3. Survival curves of fish species that experienced mortality when exposed to ranavirus isolates from three different ectothermic classes (i.e. turtle, fish and amphibian). Results are based on exposure of 20 individuals for 28 days.

APPENDIX C

CHAPTER IV TRANSMISSION OF RANAVIRUS BETWEEN ECTOTHERMIC VERTEBRATE HOSTS

TABLES AND FIGURES

Table 4.1. Average time of mortality for amphibian larvae exposed to ranavirus when housed with an amphibian, reptile, and a fish host. Direct refers to when the amphibian larvae was exposed to the virus in a water bath for three days prior to the beginning of the experiment, and indirect refers to when the counterpart host was pre-exposed to the virus, and infection occurred by aquatic transfer during the experiment.

	Amphibian		**Reptile**		**Fish**	
	Direct	**Indirect**	**Direct**	**Indirect**	**Direct**	**Indirect**
Mean	11.20	17.20	13.57	14.80	14.17	14.00
Standard Deviation	4.31	4.39	5.68	1.81	6.13	0.00
Range	14	12	18	5	18	0
Minimum	5	10	7	12	6	14
Maximum	19	22	25	17	24	14

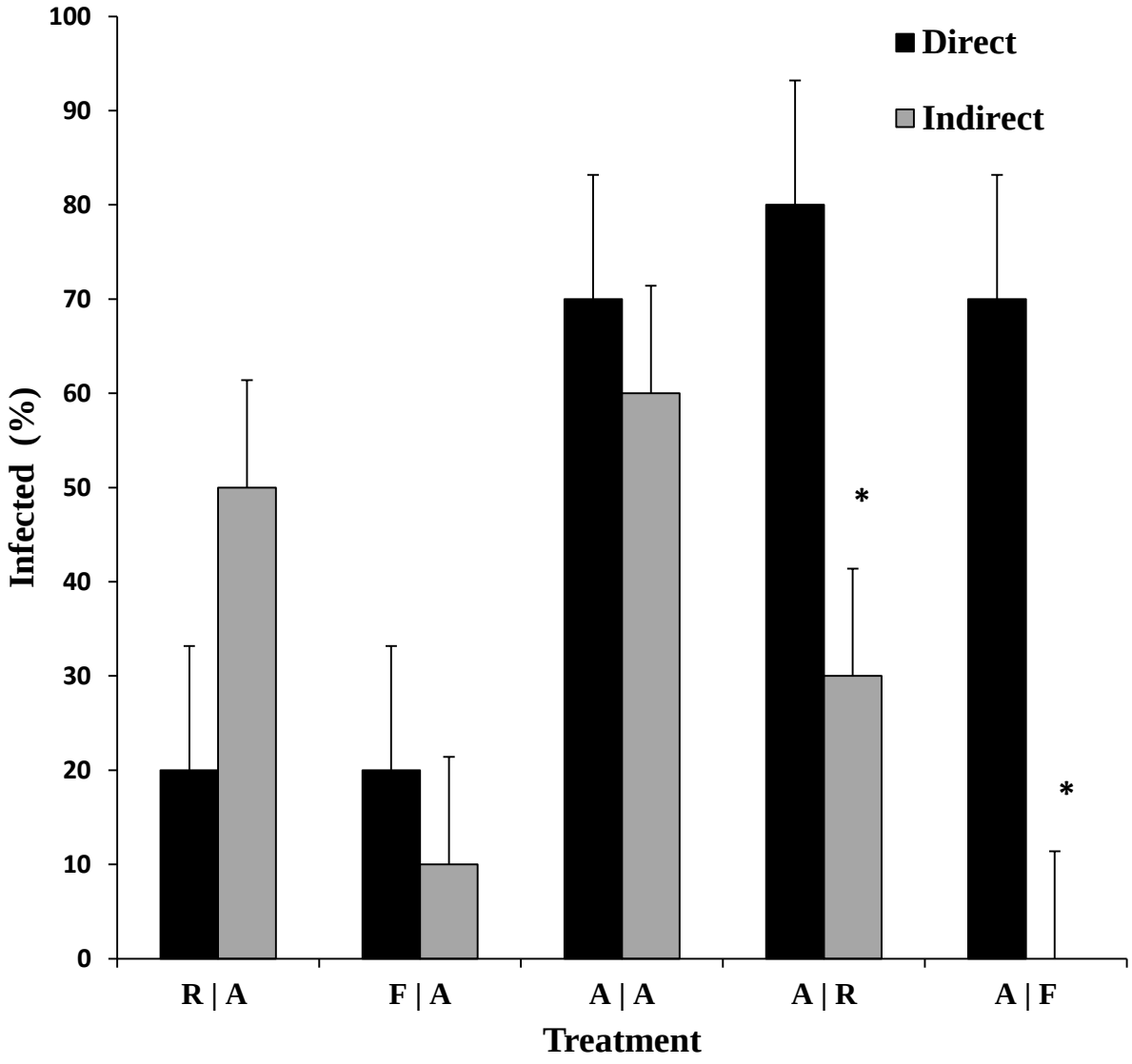

Figure 4 1. Final infection rate of amphibian larvae (A), turtles (R) and fish (F) exposed to ranavirus prior to the experiment (Exposed) or by transmission from infected counterpart (not exposed). Stars represent statistically significant differences between transmission modes ($p<0.05$). n=20 individuals of each species per treatment. Bars represent means + SE.

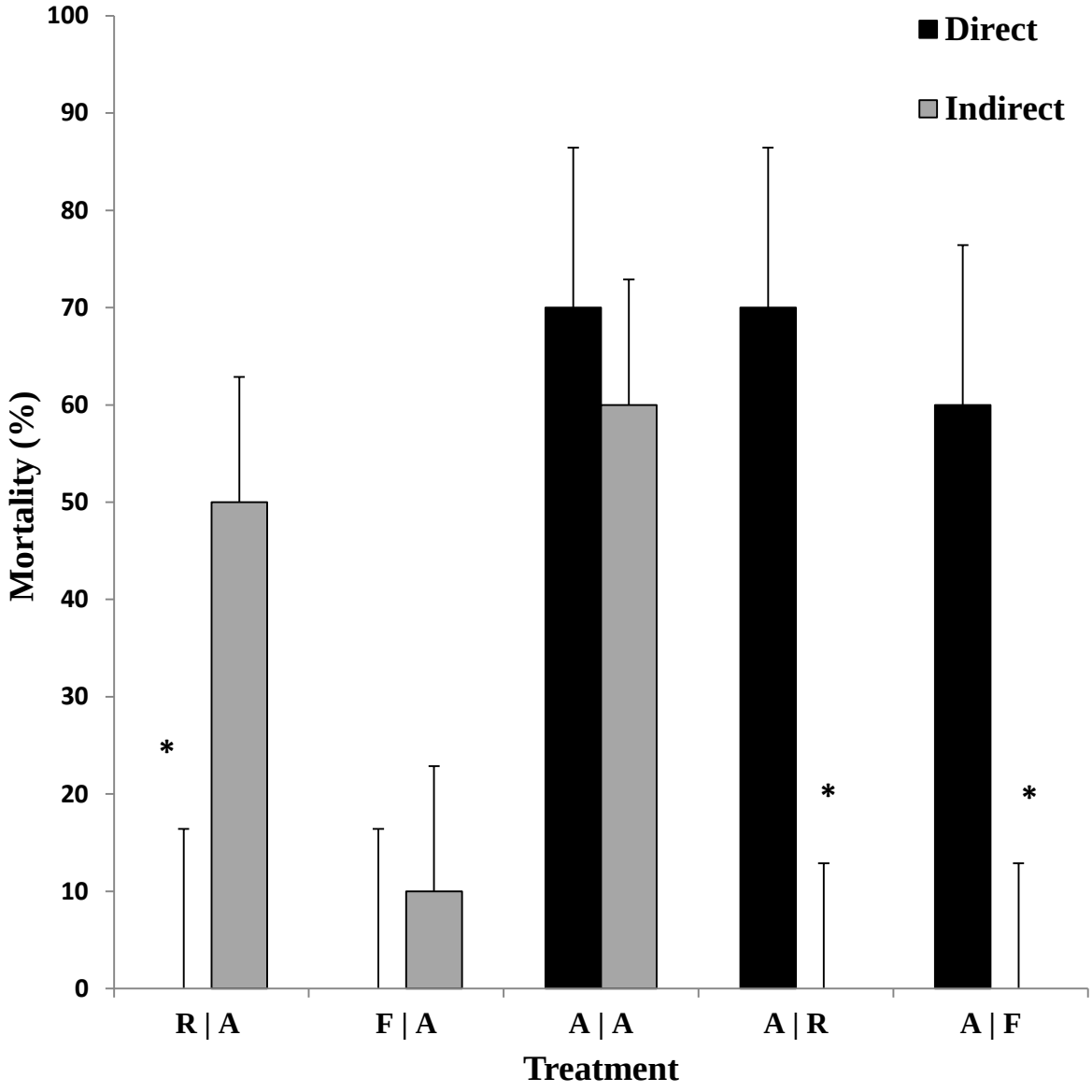

Figure 4.2. Final mortality rate amphibian larvae (A), turtles (R) and fish (F) exposed to the ranavirus prior to the experiment (Exposed) or by transmission from infected counterpart (not exposed). Stars represent statistically significant differences between transmission modes ($p<0.05$). n=20 individuals of each species per treatment. n=20 individuals of each species per treatment. Bars represent means + SE.

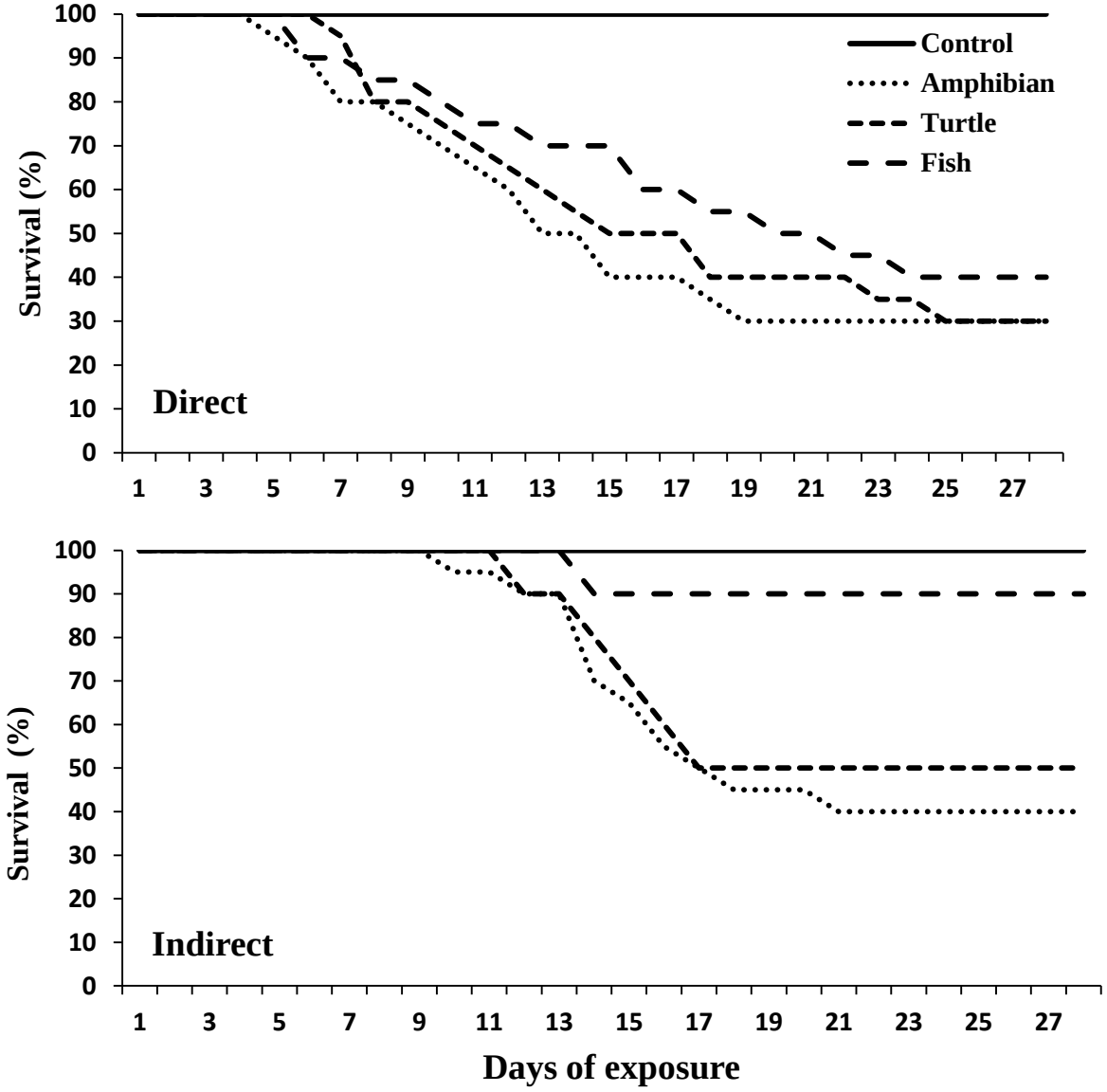

Figure 4.3. Survival curves for Gray treefrog larvae exposed to ranavirus transmitted by amphibian, reptile and fish counterparts exposed to 10^3 PFU/ml of a FV3-like ranavirus isolated from pallid a sturgeon for three days previous to the experiment. Direct= exposed previous to experiment, Indirect= exposed by housing with infected host. n=20 individuals of each species per treatment.

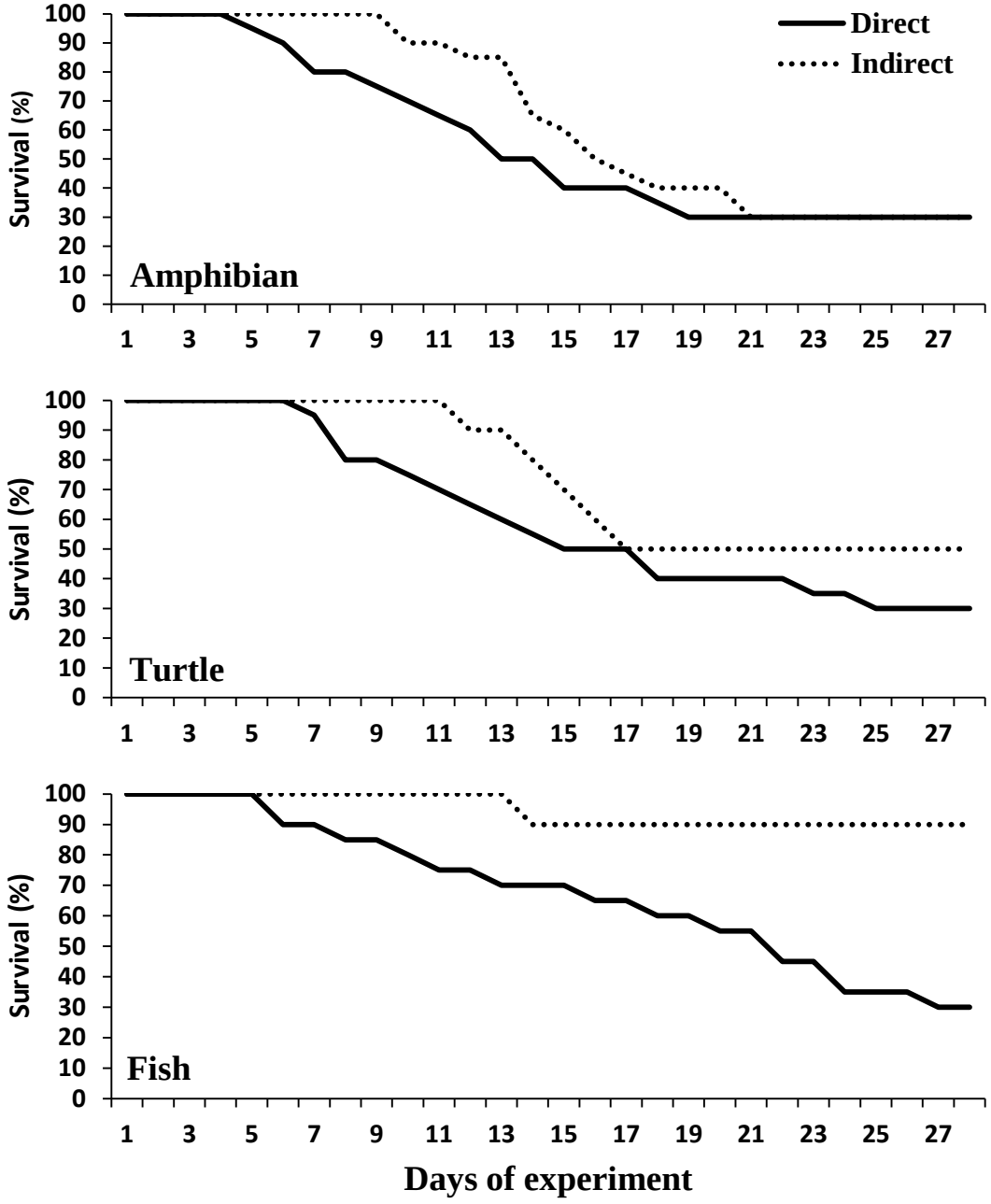

Figure 4.4. Survival curves for Gray treefrog larvae exposed to ranavirus transmitted by amphibian, reptile and fish counterparts exposed to 10^3 PFU/ml of a FV3-like ranavirus isolated from pallid a sturgeon for three days previous to the experiment. Direct= exposed previous to experiment, Indirect= exposed by housing with infected host. n=20 individuals of each species per treatment.

APPENDIX D

CHAPTER V: COMMUNITY COMPOSITION AFFECTS OUTCOME OF A RANAVIRUS OUTBREAK

TABLES AND FIGURES

Table 5.1. Percent mortality of individuals from the Appalachian community exposed to ranavirus directly by inoculation or indirectly by cohabitation with infected species. The experiment lasted 8 weeks.

	Species		
Infected species	**Wood frog**	**Chorus Frog**	**Spotted salamander**
Wood frog	100*	42	12[§]
Chorus Frog	72	44*	4
Spotted	17[§]	24	6*

*direct exposure mortality

[§] significant difference

Table 5.2. Percent mortality of individuals from the coastal plain community exposed to ranavirus directly by inoculation or indirectly by cohabitation with infected species The experiment lasted 8 weeks.

	Species		
Infected species	**Gopher frog**	**Chorus frog**	**Southern toad**
Gopher frog	100*	51	34
Chorus frog	70	78*	57
Southern toad	80	32	75*

*direct exposure mortality

§ significant difference

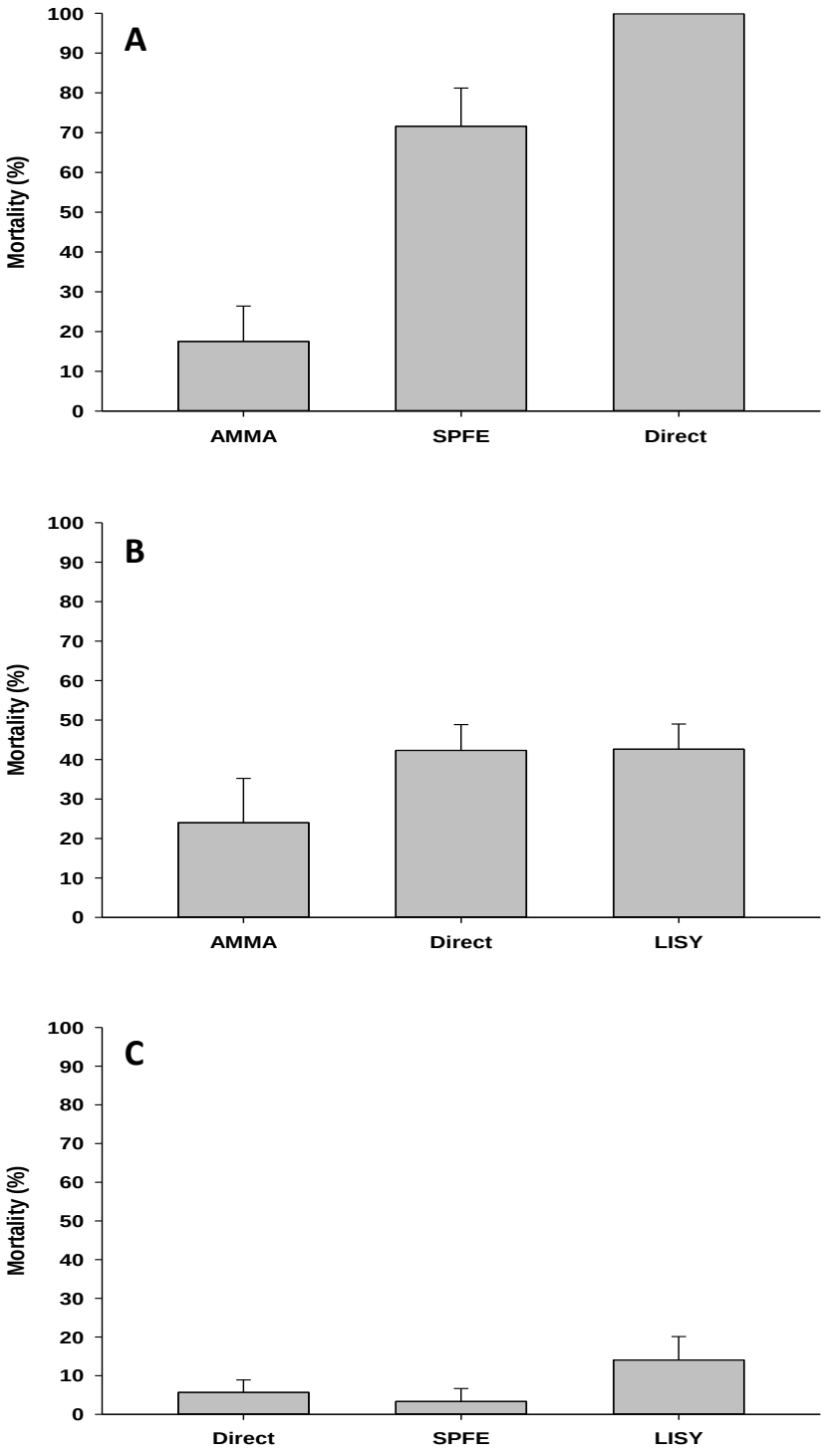

Figure 5.1. Percent mortality of individuals (A= Wood frog, B= Chorus frog, C= Spotted salamander) when exposed to the ranavirus directly by inoculation or indirectly by contact with another species; AMMA= Spotted Salamander, SPFE=Chorus frog, LISY=Wood frog. Bars represent standard error.

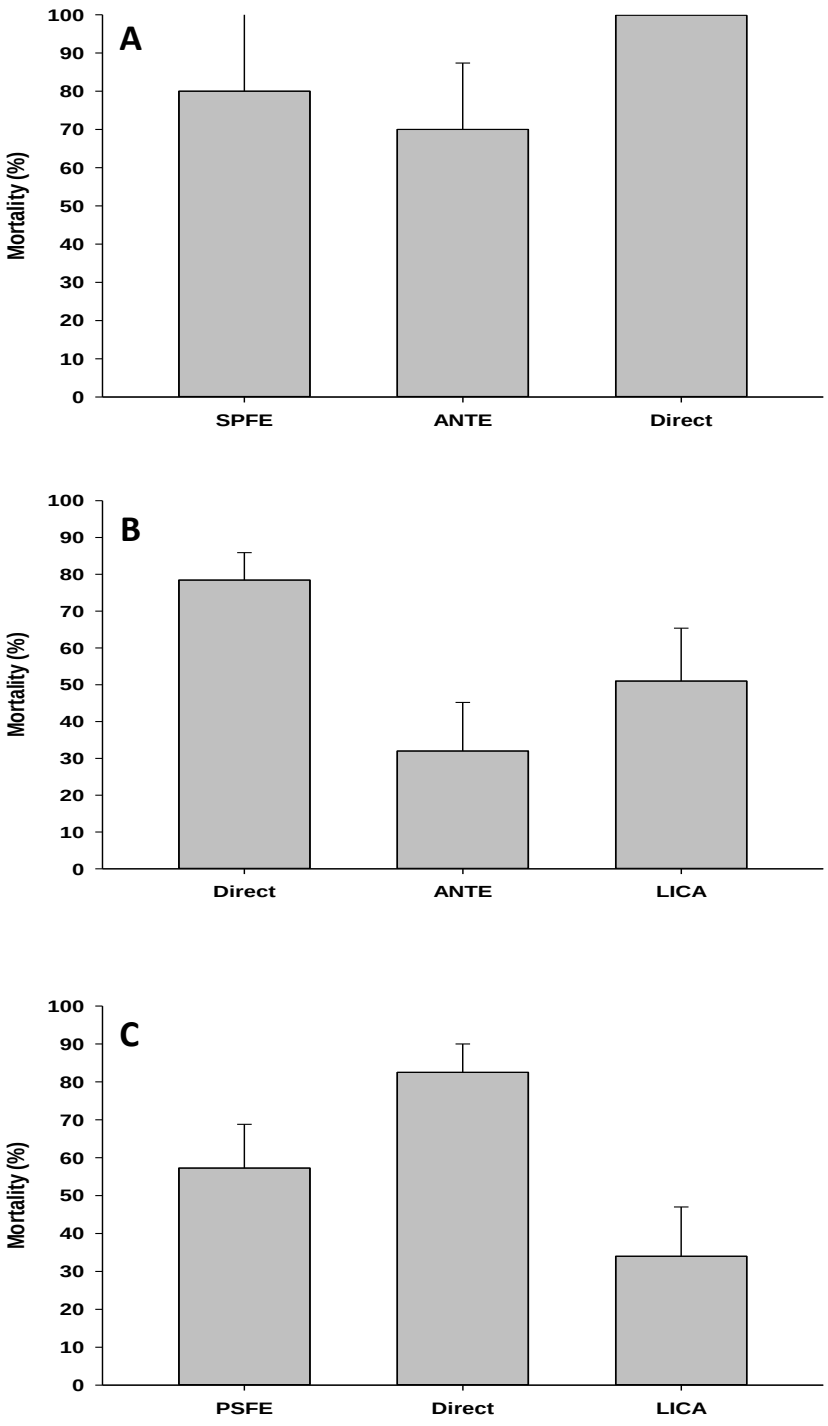

Figure 5.2. Percent mortality of individuals (A= Gopher frog, B= Southern toad, C= Chorus frog) when exposed to the ranavirus directly by inoculation or indirectly by contact with another species; SPFE=Chorus frog, ANTE= Southern toad, and LICA= Gopher frog. Bars represent standard error.

VITA

Roberto Brenes is originally from Costa Rica where he earned a bachelor degree in biology from Universidad de Costa Rica. For his M.S., Roberto studied the ecology of aquatic salamanders at the University of Texas-Tyler. Between his M.S. and Ph.D., he did research in Panama examining the ecosystem effects of amphibian die-offs associated with chytridiomycosis. In 2010, he came to the University of Tennessee (UT) to pursue his doctorate under the direction of Dr. Gray and Dr. Miller of the UT Center for Wildlife Health working with ranaviruses.