CHARACTERIZING THE EPIDEMIOLOGY OF RANAVIRUS IN NORTH AMERICAN CHELONIANS: DIAGNOSIS, SURVEILLANCE, PATHOGENESIS, AND TREATMENT

BY

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DISSERTATION

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ABSTRACT

Ranaviruses have been proposed as a major threat to amphibian biodiversity, however the impact of these pathogens on reptiles is less well understood. In this dissertation, a quantitative PCR was developed that was 100% efficient in detecting the 54 bp segment of the major capsid protein of frog virus 3 (FV3) (Genus: *Ranavirus*; Family: *Iridoviridae*). This assay was used to estimate the prevalence of ranavirus infections in 606 eastern box turtles (*Terrapene carolina carolina*) from several different states. The overall prevalence of ranaviruses in this study population was 1.5% (95% confidence interval: 0.8 – 2.9%), with a non-significantly higher prevalence in juveniles (3.5%; 95% CI: 0.9 – 11.9%) than adults (0.5%; 95% CI: 0.1 – 1.8%). The low prevalence found in this population supports the theory that this virus is associated with acute disease and death. Clinical signs recorded in box turtles that were significantly associated with ranavirus infection were fractures and diarrhea. In challenge studies at two separate environmental temperatures, red-eared sliders (*Trachemys scripta elegans*) inoculated through intramuscular injection with a FV3-like virus had higher mortality rates when maintained at 22^{o}C than at 28^{o}C, supporting the theory that this virus is less virulent at higher temperatures. FV3-like DNA was detected in whole blood, oral swabs, and cloacal swabs. The sensitivity and specificity of detection in whole blood samples when compared to necropsy were 100%, while sensitivity and specificity in oral and cloacal swabs were found to be 83% and 100%, respectively. Skeletal muscle from injection site and kidney had the highest viral copy number post-mortem, while tongue had the lowest. Significant histopathological changes included fibrinoid vasculitis in all tissues. Clinical signs observed in experimentally inoculated red-eared sliders included

lethargy, conjunctivitis, oral plaques, oral ulcers, and injection site swelling. Hematologic changes were evaluated in both free-ranging box turtles and experimentally inoculated red-eared sliders. Red-eared sliders showed only one significant change, a reduction in total solids over time. Box turtles were non-significantly lymphopenic. Intracytoplasmic inclusions were identified in two infected red-eared sliders and one infected box turtle, but they were not consistently associated with ranavirus-status. Treatment of ranavirus with anti-viral therapy has been reported to have variably poor success, but was based on anecdotal dosing recommendations. Pharmacokinetic analysis of a single oral dose of valcyclovir demonstrated measureable levels, and may prove useful against this virus. The work presented in this dissertation provides new insight into the epidemiology of ranavirus in chelonians.

Dedicated to my wife, son, and daughter

In memory of Wrigley

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TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Wildlife are subject to an array of diseases that threaten their long-term sustainability. There are a number of factors that can influence the impact of these diseases on wildlife. Alterations in the environment, including habitat degradation, fragmentation, and contamination, can have a major impact on the epidemiology of wildlife diseases, especially at the human-wildlife interface (Daszak et al., 1999; Fox, 2001; Holladay et al, 2001; Bhatia et al., 2005; Howsam et al. 2004). Furthermore, ectothermic animals, such as reptiles, are dependent on environmental temperature for many physiologic and immunologic functions (Brown and Sleeman, 2002), making them more sensitive to environmental factors than birds or mammals (Donaldson, 2005; Ultsch, 2006). Because these different factors can influence the course of a disease, it is important to evaluate them when characterizing the epidemiology of a disease in a particular species of animal.

Several terrestrial and aquatic turtles and tortoises (chelonians) are experiencing population declines across the world. The International Union for Conservation of Nature (IUCN) redlist listed 32 chelonians as critically endangered, 44 as endangered, and 58 as vulnerable in 2011. It listed an additional species as conservation dependent and 36 as near threatened (van Dijk, 2011). In 2011, 15 chelonians changed their redlist status, two were downgraded to critically endangered, four were newly listed as endangered, and four were newly listed as vulnerable; only 3 were upgraded to least concern (van Dijk, 2011). Therefore, of the 328 total species of chelonians in the world, 178 (53%) are listed with population stability as near-threatened or worse, including 8 species that are already extinct (Rhodin et al., 2010).

One species, the North American box turtle (*Terrapene carolina carolina*), has experienced significant population declines in numerous areas throughout its range, leading to its population status being downgraded in 2011 from near threatened to vulnerable (IUCN, 2011). Historically, North American box turtles have been used as sentinels of environmental health due to their wide distribution, small home range, and longevity (Sleeman, 2008, Donaldson and Echternact, 2005; Ultsch, 2006). A product of these investigations are reports of free-ranging box turtles being afflicted with different diseases, both infectious and non-infections in nature, that are affecting their population stability (Sleeman, 2008, Donaldson and Echternact, 2005; Ultsch, 2006).

Specific causes for the peril of box turtles have been attributed to human-induced factors including: road and mowing mortality, collection for the pet trade, nest depredation, prescribed burning, disturbance of nest sites by off-road vehicles, and habitat loss (Green et al., 2007; Lee, 2007; Tarasan and Delis, 2007; Nazdrowicz et al., 2008). Habitat encroachment and climate change can affect resource availability, which has been shown to delay sexual maturity and alter life cycle characteristics (Ernst et al., 1994). These types of negative impacts are especially detrimental to long-lived, low fecund animals and could prevent population recovery from catastrophic disturbances, such as disease outbreaks (Budischak et al., 2006; Dodd and Dreslik, 2008). Therefore, the impacts of anthropogenic changes, such as global climate changes, suburbanization, and habitat fragmentation, may be considerable for box turtle populations and are worth further investigation.

While a combination of factors is likely playing a role in the population declines of the box turtle, disease has been emerging across the eastern USA in chelonians. There have been several investigations into the health of box turtles, including recent retrospective studies in

Virginia and Tennessee (Brown and Sleeman, 2002; Schrader et al., 2010). These and other investigations have identified trauma, aural abscessation, organochlorine exposure, mycoplasmosis, and *Ranavirus* infection as historic and current threats to box turtles (Brown and Sleeman, 2002; Schrader et al., 2010; Allender et al., 2006, 2011; Feldman et al.; Holladay et al., 2011). Ranavirus has been emerging in several chelonian species across the United States (Allender, 2011 JMIH abstract). It is imperative to further evaluate morbidity and mortality events in box turtles to determine the cause and prevalence of this and other diseases and their potential role(s) in population declines.

Several species of ranaviruses have been isolated during disease outbreaks. Since 1991, over 70 epizootics involving ranaviruses in reptiles and amphibians have been reported in the literature (as summarized in this dissertation). These outbreaks have occurred throughout the world including North America (New Hampshire, Colorado, North Dakota, Minnesota, Utah, Maine, Idaho, Tennessee, Massachusetts, Wyoming, North Carolina, Arizona, Georgia, Florida, Pennsylvania, New York, Texas, Kentucky, Saskatchewan, Ontario), South America (Brazil, Venezuela, Uruguay), Europe (Croatia, United Kingdom, Spain, Portugal, Denmark, Germany), Asia (China, Japan), and Australia. The type species of the genus is frog virus 3 (Granoff, 1966); it is the only ranavirus shown to infect chelonians.

This dissertation aims to address key gaps in the epidemiology of ranaviral disease in chelonians. The following objectives will be presented:

1. Develop a quantitative (real-time) polymerase chain reaction (PCR) assay for detection of frog virus 3-like DNA in chelonians.
2. Determine prevalence of frog virus 3-like virus in free-ranging eastern box turtles presented to rehabilitation and non-rehabilitation centers in the southeastern US.

3. Determine the clinical pathologic response to experimental frog virus 3-like infection in a red-eared slider (*Trachemys scripta*) model.
4. Determine the survival rate and pathologic response to experimental frog virus 3-like infection in a red-eared slider (*Trachemys scripta*) model.
5. Determine the earliest time of detection and progression of viral load of frog virus 3-like virus in whole blood, oral swabs, and cloacal swabs using the qPCR in a red-eared slider (*Trachemys scripta*) model.
6. Establish the pharmacokinetic profile of a single dose of oral valcyclovir in healthy box turtles.

CHAPTER 2

LITERATURE REVIEW

Background

Wildlife diseases have been on the rise across the world (Daszak et al., 1999). While some of these diseases receive a great deal of attention, such as of chytridiomycosis in worldwide amphibian declines, white-nose syndrome in North American bats, West Nile virus in birds, and Chronic Wasting Disease in cervids (Daszak et al., 1999; Conner et al., 2008; Lorch et al., 2011), others are poorly understood and require further study. Disease events can have a dramatic impact on local populations, increasing as population size decreases (Daszak, 1999). Alarmingly, these disease events are likely underreported (Daszak, 1999) due to a lack of awareness, few long-term research studies, and a lack of disease monitoring in biological studies. Reptiles are not exempt from these factors, and often times there are fewer disease reports in these species than mammals or birds. To fully appreciate the role of disease on wild reptile populations it is important to characterize the epidemiology of those diseases we do recognize. A disease that has been reported in numerous reptile species that possibly leads to population declines in herptiles and warrants further study are from the family *Iridoviridae*.

The family *Iridoviridae* consists of five genera and several unclassified species (ICTV, 2011). *Chloriridovirus* and *Iridovirus* are known to infect invertebrates, while *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus* affect ectothermic vertebrates including fish, amphibians, and reptiles (ICTV, 2011). They are large, icosohedral, DNA viruses that can be found in an enveloped or non-enveloped form. Diseases caused by viruses in the genera *Ranavirus* are a

growing concern in aquaculture, as well as free-ranging amphibian and reptile populations (Chinchar, 2002). There are six recognized species in the genus Ranavirus including frog virus 3 (FV3), *Ambystoma tigrinum* virus (ATV), Bohle iridovirus (BIV), Epizootic hematopoietic necrosis virus (EHNV), European catfish virus (ECV), and Santee-Cooper ranavirus (ICTV, 2011). These viruses are found worldwide and cause significant morbidity and mortality in affected species (Williams et al., 2005). Recently, ranaviral disease was placed on the World Organization for Animal Health list of reportable diseases for amphibians (OIE, 2012).

There have been several reviews of iridoviruses, as well as reviews of amphibian and reptile diseases (Williams et al., 2005; Densmore and Green, 2007; Chinchar, 2009; Gray et al., 2009; Duffus et al., 2010; Robert, 2010); however, there is a paucity of information on reptile iridoviruses. Because of these limitations, we must rely on knowledge of this virus in fish and amphibians to elucidate general virus ecology to help frame current and future research in reptiles.

Epidemiology

The discovery of FV3 was first reported in leopard frogs being studied for Lucke's renal adenocarcinoma in the 1960's (Granoff, 1966). Since that time numerous outbreaks have occurred in both free-ranging and captive herpetofauna across the world (Table 2.1). Today, active monitoring of both free-ranging and captive animals is occurring on nearly every continent.

Since 1991, over 80 epizootics involving ranaviruses in reptiles and amphibians have been reported in the literature (Table 2.1). These outbreaks have occurred throughout the world, including North America (New Hampshire, Colorado, North Dakota, Minnesota, Utah, Maine,

Idaho, Tennessee, Massachusetts, Wyoming, North Carolina, Arizona, Georgia, Florida, Pennsylvania, New York, Texas, Kentucky, Saskatchewan, Ontario), South America (Brazil, Venezuela, Uruguay), Europe (Croatia, United Kingdom, Spain, Denmark, Germany, Netherlands), Asia (China, Japan), and Australia.

Various ranaviruses accounted for the epizootics, including FV3, ATV, soft-shelled turtle virus (STIV), BIV, and as of yet unclassified iridoviruses. FV3-like virus is the only virus identified in turtles in North America, and is also the most commonly reported iridovirus for anurans. ATV mainly affects salamanders in the western US, while BIV is the main ranavirus identified in Australia (Greer et al., 2009; Ariel, 1997). Outbreaks in amphibians have accounted for anywhere between 1 and 600 deaths during each outbreak, accounting for up to 90% mortality of a single population (Miller et al., 2011).

A majority of the reported epizootics have involved amphibians, which include 70 species from the following fourteen families: *Ranidae, Bufonidae, Hylidae, Dendrobatidae, Plethodontidae, Cryptobranchidae, Hynobiidae, Myobatrachidae, Salamandridae, Alytidae, Ambystomatidae, Rhacophoridae, Scaphiopodidae, Leptodactylidae* as summarized in Miller et al., (2011). High mortality is reported for most species during these outbreak investigations. Roughly 60% of the outbreaks involved larval amphibians, 30% involved adults, and 10% involved recent metamorphs (Green et al., 2002; Gray et al., 2009; Miller et al., 2011).

Unfortunately, there has been a rise in the number of reports and cases in chelonians, and specifically box turtles (*Terrapene* sp.), in the US (Johnson et al., 2008; Allender et al., 2006, DeVoe et al., 2004). The cause of these increases is unknown, but may be due to an increased incidence or awareness, or improved testing methods and increased surveillance. Reptile species involved in these outbreaks include eastern box turtles (*Terrapene carolina carolina*), gopher

tortoises (*Gopherus polyphemus*), soft-shelled turtles (*Trionyx sinensis*), Burmese star tortoises (*Geochelone platynota*), green tree pythons (*Morelia viridis*), and leaf-tailed geckos (*Uroplatus fimbriatus*). Reptile outbreaks have mainly involved adult animals (Johnson, 2006). Box turtles have been implicated in ten of the outbreaks, all occurring within North America (Allender et al., 2011b; DeVoe et al., 2004; Allender et al., 2011a; Johnson et al., 2008; Ruder et al., 2008; Johnson et al., 2010). Eastern box turtles have been observed frequently associated with ranavirus outbreaks and should be investigated. While eastern box turtles are primarily terrestrial, they have been shown to spend a considerable amount of time in temporary ponds (Donaldson and Echternacht, 2005), which may expose them to ranavirus through water. Additionally, juvenile turtles may be at higher risk based on other transmission studies and warrant further investigation.

Ranaviruses have a wide species distribution, with significant differences in species susceptibility even within the same isolate (Schock et al., 2008; Hoverman et al., 2010). There are several species that appear uniquely sensitive to these viruses, including tiger salamanders to ATV (Greer et al., 2009), wood frogs to FV3 (Greer et al., 2005; Harp and Petranka, 2006; Schock et al., 2008), and eastern box turtles to FV3 (Allender et al., 2006; Johnson et al., 2006; Ruder et al., 2010).

Many reports indicate that larval amphibians in North America are more susceptible to mortality than adults, whereas adults are more commonly reported in European amphibians (Hoverman et al., 2010; Miller et al., 2011; Duffus et al., 2010; Robert, 2010). In Dybowski's frogs, both adults and tadpoles demonstrated mortality, although mortality in tadpoles (42.5%) was higher than adults (5.7%). The mechanisms for the difference in mortality based on age/ life stage is unknown, but may be associated with undeveloped immune responses, entirely aquatic

lifestyles, feeding habits, or behaviors that result in density-dependent transmission (Robert, 2010).

Adult chelonians have been more commonly reported to develop FV3-like infections than juveniles (Johnson, 2006). However, there are fewer overall reports in reptiles with FV3-like than amphibians, leading to the likelihood that adult reptiles are more likely sampled and diagnosed than juveniles. A current survey in eastern box turtles demonstrated a significant association between FV3-like positive animals and age (juveniles) (Allender et al., 2012a). In Australia, juveniles of two species of tortoise (*Emydura krefftii* and *Elseya latisternum*) were susceptible to BIV, while adult tortoises, juvenile crocodiles, and three species of snakes were not affected after experimental transmission (Ariel, 1997). Despite the lack of clinical signs, isolation of BIV was successful in an adult brown tree snake and indicates a permissible reservoir host (Ariel, 1997). Therefore, it is likely to consider that other ranaviruses, including FV3, may display the same juvenile predilection (e.g. high juvenile mortality and possible adult carriers) in reptiles that is observed in amphibians.

Disease events in amphibians are often clustered into local epizootics, with significant impact on local populations (Miller et al., 2011). These epizootics have been scattered across numerous habitats and landscapes in the USA, and in some cases have occurred on an annual basis (Fox et al., 2006; Carey et al., 2009; Greer et al., 2009). In a multi-year survey of ponds that experience recurrent ATV outbreaks in tiger salamanders, prevalence ranged from 0% in one year to 57% in other years (Greer et al., 2009). Unfortunately, disease predictability outside these annual occurrences has not been successful. The effect of population stability in the face of ranavirus outbreaks has been debated. Several reports have concluded that ranaviruses are a threat to biodiversity within an ecosystem (Pearman et al., 2005; Gray et al., 2009; Teacher et al.,

2010; Miller et al., 2011), while others suggest it can have a local population impact but does not lead to extirpation (Greer et al., 2008). In a retrospective analysis of population stability and ranavirus outbreaks in the UK, the authors observed an 81% decline in the number of adult frogs in infected ponds from 1996 to 2008, while comparative uninfected ponds showed no change (Teacher et al., 2010). Utilizing these same populations, the authors found that ranavirus infection could affect mate selection behavior, resulting in decreased relatedness and elevated homozygote excess, and concluded that this could serve as a detrimental, non-lethal impact of ranavirus infection on these amphibian populations (Teacher et al., 2009). During a transmission study of ATV in tiger salamanders, a species that has experienced significant mass mortality events in the western USA, it was observed that population density was a significant predictor of mortality (Greer et al., 2008). Animals in higher density populations had higher mortality rates and died more quickly, however, it was also observed that extinction events were unlikely because as mortality reduced density below a critical threshold remaining animals were able to recover (Greer et al., 2008). This has been supported by field studies in Arizona (USA) that demonstrate a high number of salamander deaths, but not local extinction (Greer et al., 2009). In addition to lethal effects on amphibians, developmental instability (a measure of stress and overall quality of organisms) is more frequently observed in adult frogs, indicating possible non-lethal effects on population stability (St-Amour et al., 2010). These examples suggest that the impact of the disease may be local or regional, and that it is a complex, multifactorial system that determines outcome. More research is needed to further elucidate the impact and role of this virus on amphibians. Investigations of landscape factors that increase ranavirus pathogen prevalence indicate that within-pond stressors are more important than vector movement (Gahl et al., 2008). Furthermore, high catchment (drainage basin) and headwater wetlands such as vernal

ponds were significantly associated with ranavirus larval mortality events (Gahl et al., 2008). Water conditions, including aluminum concentrations, temperature, and conductivity, have also been found to be associated with ranavirus outbreaks, but it was unclear if they increased the mortality events or were just associated with an outbreak (Gahl et al., 2010). The authors of that study concluded that each wetland will have a unique set of biological, chemical, and physical stressors that lead to mortality events (Gahl et al., 2010). Other authors have also found that environmental factors can have an impact on the epidemiology of ranavirus in amphibians. Greer and Collins (2008) found that ponds with less vegetation had higher prevalence rates; this was attributed to higher densities of larval amphibians at the edge of ponds seeking shelter and thereby increasing density-dependent transmission of the virus. While that study was done under natural conditions, experimental transmission of the virus did not find similar results regarding animal density. Harp and Petranka (2006) found that mortality was > 98% in wood frogs infected with ranavirus under both laboratory and field mesocosm settings regardless of animal density. Virulence of the virus likely plays an important role regarding the epidemiology of the virus and was not evaluated in these two studies. Wetland restoration is a common technique to mitigate the effects environmental degradation, but long-term monitoring of these wetlands is lacking for most sites beyond 3 to 5 years (Petranka et al., 2006). During an eight-year study comparing reconstructed and natural ponds, ranavirus was significantly associated with increased juvenile survival that threatened long-term sustainability (Petranka et al., 2006). As more is learned about the epidemiology of this virus and long-term studies are performed on different cohorts, it will be possible to further decipher the role of landscape factors on the pathogenicity of this virus in amphibians.

Most ranavirus surveillance programs utilize pathogen-based (PCR, virus solation) assays to confirm the presence or absence of the organism in an animal or its environment (Gray et al., 2009); however, certain studies have utilized serologic (e.g., enzyme-linked immunosorbent assays [ELISA]) assays to determine exposure (and recovery) from ranaviruses in some animals (Johnson et al., 2010). Serologic surveys of ranaviruses in reptiles have been performed in only two studies (Ariel, 1997; Johnson et al., 2010). Both of these reports utilized ELISA to determine exposure in juvenile and adult animals. In a serologic survey of 1000 gopher tortoises in the southeast USA, the seroprevalence of FV3-like virus was 1.5% (95% confidence intervals [CI]: 0.5-2.2) (Johnson et al., 2010). In that same study, the prevalence of FV3-like virus in 55 box turtles was 1.8% (95% CI: 0.7-2.9) (Johnson et al., 2010). The authors suggested that antibody response against FV3 is low in North American turtles because the course of disease is short and the animals die before developing an effective and measureable antibody response. However, several species of adult reptiles tested in Australia demonstrated high levels of exposure, including tortoises (Ariel, 1997). The assay used in the Australian study identified exposure as reactivity to BIV-like proteins in the ELISA and not fully definitive for antibody presence (Ariel, 1997). The low seroprevalence noted in the gopher tortoises and box turtles may have also been associated with the misidentification of the animals. The sensitivity and specificity of these assays was not described, but if the specificity of the assay was <96%, it is possible that the animals characterized as positives could have been false positives. This is important to consider because the theory that the seroprevalence was low because of the acute nature of the disease is based on these results, when in fact this may not be true if the animals were misclassified. Additional work is required to further determine the value of these assays in characterizing the status of exposed/infected reptiles.

Serologic surveys in amphibians are less common, but have demonstrated antibodies in the giant toad (*Bufo marinus*) on two continents (Zupanovic et al. 1998b). However, the significance of the findings is unknown in light of the fact that antibodies are not routinely produced early in infections and likely do not reflect the true prevalence.

Several of the reports of FV3-like infections in reptiles have been in captive animals (Marschang et al., 1999; DeVoe et al., 2004; Benetka et al., 2007; Johnson et al., 2008) (Table 2.2). FV3-like infections were described in two juvenile diseased Hermann's tortoises (*Testudo hermanni*) in Germany using PCR in which all seven animals in the group died with similar signs (Marschang et al., 1999). These tortoises were described with systemic disease that was characterized as hyperemia and ulcerations of the oral cavity of undetermined duration (Marschang et al., 1999). A leopard tortoise (*Geochelone pardalis pardalis*) with nasal discharge, stomatitis, and lethargy had a concurrent ranavirus and herpesvirus infection confirmed by PCR (Benetka et al., 2007). The animal received supportive care and recovered after four months (Benetka et al., 2007). A group of ten juvenile green tree pythons with chronic ulceration of the nasal mucosa, pharyngeal edema, and necrosis of the liver had a ranavirus isolated from pooled necropsy tissues that was 97% homologous to FV3 (Hyatt et al., 2002). A leaf-tailed gecko with granulomatous glossitis and hepatitis died unexpectedly and was diagnosed with a ranavirus (related to FV3) infection based on EM, restriction endonuclease assay, and sequencing (Marschang et al., 2005). Captive farmed reptiles are increasing in popularity in China, especially as a food source, and reports of ranavirus infections in soft-shelled turtles are on the rise (Chen et al., 1999).

Farmed amphibians are common in many countries, and recent outbreaks of ranaviruses have occurred in leopard frogs (*Rana pipiens*) in Brazil and Uruguay and Chinese giant

salamanders (*Andrias davidianus*) and pig frogs (*Rana grylio*) in China (Zhang et al., 2001; Galli et al., 2006; Mazzoni et al., 2009; Geng et al., 2011). Additionally, American bullfrogs (*Lithobates catesbeianus*) have suffered high mortality rates from ranavirus infections in a ranaculture facility in the US (Miller et al., 2007) and in Japan (Une et al., 2009). Larval or recently metamorphosed frogs were affected in both outbreaks (Miller et al., 2007; Une et al., 2009). In Japan, the outbreak occurred over 45 days and included several thousand animals (Une et al., 2009). These patterns of age structure and short disease duration with high mortality are consistent with free-ranging amphibian outbreaks.

Reptiles have also been infrequently observed with unclassified or other genera of iridoviruses. These include a captive collection of moribund chameleons (*Chamaeleo quadricornis* and *Chamaeleo hoehnelli*) with an iridovirus that was characterized using electron microscopy (Drury et al., 2002). A case series involving two bearded dragons (*Pogona vitticeps*) and a chameleon (*Chamaeleo quadricornis*) identified an iridovirus with 97% homology to Chilo iridescent virus (genera *Iridovirus*) (Just et al., 2001). While these iridoviruses have not been associated with free-ranging reptile diseases, similar pathogenesis or immune response to these viruses may help to characterize the epidemiology of other iridoviruses, including ranaviruses, in reptiles.

Additionally, there are several iridoviruses that have been observed within erythrocytes of reptiles; however, the clinical and epidemiological importance of these findings is unknown. A single fer de lance (*Bothrops asper*) with renal carcinoma had intraerythrocytic inclusions in conjunction with a regenerative anemia (Johnsrude et al., 1997). Acidophillic inclusions within erythrocytes were observed in nine flap-necked chameleons (*Chamaeleo dilepsis*) and a single Fischer's chameleon (*Bradypodion fischeri*) in Tanzania (Telford and Jacobson, 1993). And

recently, a wild peninsula ribbon snake (*Thamnophis sauritus sackenii*) was observed with two types of erythrocytic inclusions that were identified as a distinct member of the *Iridoviridae* family based on PCR of the DNA polymerase gene segment that was amplified (Wellehan et al., 2008). Further research is needed to determine the role of erythrocytic iridoviruses in epidemiology in captive and free-ranging reptiles.

Several reports exist in which surveillance of reptile and amphibian populations failed to demonstrate molecular evidence or exposure to ranavirus and are summarized below. Several species of frogs were negative for ranavirus exposure using ELISA on Dominica and Montserrat (Garcia et al., 2007). Additionally, non-clinical painted turtles (*Chrysemys picta*) and Blanding's turtles (*Emydoidea blandingii*) did not show any PCR evidence of FV3-like virus (Allender et al., 2009. A survey in Colorado that detected a high prevalence of chytridiomycosis in several hundred amphibians, failed to identify a single ranavirus positive case using culture or histopathology (Green and Muths, 2005). A survey of 111 amphibians from four different species collected in Pennsylvania found only a single positive case using virus isolation and PCR (Glenney et al., 2010). A survey of 300 anurans from Jersey Island, UK was done in an attempt to characterize ongoing declines of amphibian populations, and while ranaviruses were tested using PCR as part of the protocol, none were found (Loras et al., 2011). Studies in tropical areas have occurred with less frequency, but a study in Costa Rican amphibians failed to detect ranavirus in any sample (Picco et al., 2007). More cases of negative ranavirus results likely exist, but are not reported. These reports can be used to demonstrate that while ranavirus infections have been reported globally, there remain populations that are unaffected. The testing strategies used in these studies were based on confirming the presence of the pathogen (e.g., culture or PCR), and we would expect negative status in non-clinical animals. One exception is the study in

Jersey Island, UK where population declines were noted. The results from that study should affirm that amphibian declines can be associated with a number of different pathogens and/or environmental issues. These results also further support that affected free-ranging animals are likely lost to the disease before being detected, reinforcing that this virus is associated with an acute disease process. Even in cases where captive animals are affected, clinical course of disease may be short with minimal signs, therefore it is even more critical that rapid diagnostic strategies are utilized in widespread assessments of vulnerable amphibian and reptile populations.

Clinical signs and Pathology

Amphibians, fish, and reptiles infected with ranaviruses share several common clinical and pathologic characteristics, including systemic disease with epithelial necrosis. While many reported infections lead to death prior to any premonitory signs (Gray et al., 2009; Miller et al., 2011), there are examples of antemortem clinical signs described in all of the lower vertebrates. Fish with largemouth bass virus (LMBV) infections typically develop ulcerations of the skin and muscle (Deng et al., 2011). Skin ulcerations are also commonly seen in amphibians (Miller et al., 2011; Cunningham et al., 2007b), although amphibians may also demonstrate systemic hemorrhages in the absence of skin lesions (Cunningham et al., 1996). Adult salamanders can develop abnormal swimming patterns and subcutaneous edema, while loss of pigmentation, lordosis, epithelial sloughing, and petechiation are reported in frogs (Tweedell and Granoff, 1968; Docherty et al., 2003; Miller et al., 2011).

In larval amphibians, erythema at the base of the tail, ventrum, and legs, as well as swelling of multiple areas of the body have been observed (Gray et al., 2009a). Ecchymosis and

petechiation of the skin are common (Miller et al., 2011). The gall bladder may also be enlarged, but this is attributed to anorexia (Miller et al., 2011).

Chelonians commonly develop nasal, ocular, and oral discharge, in addition to oral plaques (Allender et al., 2006; Ruder et al., 2010). Epithelial lesions are also observed in reptiles, but with less consistency (DeVoe et al., 2004). Poxvirus-like lesions have been described in bearded dragons and chameleons (Just et al., 2001). While a wide variety of clinical signs have been observed in reptiles, no study has investigated possible associations between clinical signs and infection.

Pathologic findings in susceptible individuals are similar between adults and juveniles of both amphibians and reptiles (Miller et al., 2011; Johnson et al., 2007). Infections lead to systemic organ failure due to necrosis of epithelial cells of the spleen, liver, kidney, and intestines (Miller et al., 2009; Miller et al., 2011; Johnson et al., 2007). Hemorrhagic syndromes are common in anurans in the UK (Cunningham et al., 2007a) and in salamanders in western North America (Bollinger et al., 1999). Tiger salamanders (*Ambystoma tigrinum*) have been observed with polypoid lesions early in the course of the disease that progress to cover most of the body (Jancovich et al., 1997). There are also non-specific changes noted with ranavirus infections, such as lymphocytosis, lymphoid depletion, and vacuolation of hepatocytes and renal tubular cells, that may be confused with other diseases (Gray et al. 2007; Miller et al., 2009). Basophilic viral inclusions have been inconsistently observed in affected tissues (Johnson et al., 2007; Gray et al., 2009a). When present, inclusions have been identified in erythrocytes, leukocytes, epithelial cells, meninges, gills, neuroepithelium, nasal tissues, adipose, trachea, muscle, and osteoclasts (Allender et al., 2006; Miller et al., 2011, Gray et al., 2009a; Docherty et al., 2003; Jancovich et al., 1997).

Genetic variation

Ranaviruses infect fish, amphibians, and reptiles across the world. The relationship between different ranaviruses is still poorly known, but has recently been elucidated in amphibians (Jancovich et al., 2009). Ranaviruses are large double stranded DNA viruses (105 kbp) that encode 98 open-reading frames with a GC content of 55% (He et al., 2002; Tan et al., 2004). Phylogenetic analysis has mainly concentrated on evaluation of major capsid protein (MCP) sequences (Mao et al., 1997; Jancovich et al., 2005), although most analyses have demonstrated very little genetic divergence based on the MCP (Jancovich et al., 2003; Jancovich et al., 2005). However, when other techniques such as RFLP and cell culture growth characteristics are used, differences are revealed in some cases that highlight that MCP alone is insufficient to characterize virus isolates (Majji et al., 2006). Studies using multiple gene segments and dotplot analysis have suggested that fish to salamander isolates are more closely related than salamander to frog isolates (Jancovich et al., 2009).

Recently, whole genome alignments were performed using six ranavirus (ATV, EHNV, FV3, TFV, SGIV, and GIV) isolated from fish, amphibians, and reptiles (Jancovich et al., 2009). Based on this analysis, two subspecies of ranaviruses were proposed: Grouper Iridovirus (GIV)-like viruses and amphibian-like ranaviruses that include FV3 and ATV (Jancovich et al., 2009). Branch lengths within the amphibian ranaviruses are relatively short, indicating recent speciation (Jancovich et al., 2009). Using dotplot analysis, it was demonstrated that EHNV was colinear with ATV, but not with FV3 or TFV (Tiger Frog virus) (Jancovich et al., 2009).

Because ranaviruses within the amphibian-like ranavirus subspecies includes a fish species (ENHV), and that distantly related iridoviruses infect fish, it is likely that the common ancestor was a fish virus (Jancovich et al., 2009). There are two theories on how host shifts have

led to a jump from fish to amphibians (Jancovich et al., 2009). One theory suggests that there were two separate host shifts, one from fish to salamanders and another from fish to frogs (Jancovich et al., 2009). The other theory argues that the host shift to salamanders occurred in the common ancestral virus (Jancovich et al., 2009). Based on this theory, the host shift from amphibians would require a host shift back to fish (EHNV) and frogs (FV3-like) using the current taxonomy (Jancovich et al., 2009). All scenarios also demonstrate a host shift from frogs to turtles, as isolates from frogs are colinear with turtle isolates (Jancovich et al., 2009). Sequence analysis of soft-shelled turtle iridovirus (STIV, a ranavirus) demonstrated that it was identical to FV3, indicating a co-evolution (Huang et al., 2009).

It is speculated that these host jumps have occurred within the last 40,000 years due to an increase in human harvesting of aquatic organisms (Jancovich et al., 2009). Analysis of intraspecific phylogeny further revealed that human introduction of viruses is a possible mechanism for disease emergence (Storfer et al., 2007). Whole genome analysis has demonstrated numerous host shifts: fish to salamander, fish to frogs, and frogs to turtles (Jancovich et al., 2009). This reinforces the occurrence of ranaviruses in several species of ectothermic vertebrates and increases the likelihood that more host shifts will occur (Jancovich et al., 2009). Understanding the phylogeny of these viruses is crucial to management of this group of viruses, as its host range is larger than any other group of viruses (Jancovich et al., 2009).

Other conserved gene segments have been less frequently used to establish phylogeny. One of those gene segments, thymidine kinase (TK), is a component of several large DNA viruses, including herpesviruses, poxviruses, and iridoviruses (Coupar et al., 2005). Sequences of TK genes were compared among several ranaviruses and showed similar homology (Coupar et al., 2005).

Molecular Characteristics

The 105 kbp FV3 genome encodes 98 non-overlapping open reading frames (ORF). These ORF lead to the appearance of immediate early (IE), delayed early (DE), and late (L) viral transcripts (Majji et al., 2009). IE and DE genes are responsible for nucleic acid metabolism and immune evasion, while L genes have been responsible for DNA packaging and virion assembly (Chinchar et al., 2009; Majji et al., 2009).

The cellular receptor (specific receptor not yet identified) for entry of FV3 is highly conserved among many vertebrate cell lines, including those derived from mammals, birds, herptiles, and fish (Chinchar et al., 2009). However, *in vivo* hosts are restricted to ectotherms, in part because temperatures above 32°C do not allow viral replication, and/or these receptors are not widely represented in endothermic vertebrate hosts (Chinchar et al., 2009).

Ranaviruses have both enveloped and non-enveloped virions that are produced during infections, and both are infective. Non-enveloped virions enter the cell by binding to plasma membrane and injecting viral particles into the cytoplasm, whereas enveloped virions enter the cell via receptor-mediated endocytosis. Enveloped virions are more infective, which may be due to at least two different host receptor proteins (Williams et al., 2005).

Viral DNA (enveloped virions) is translocated from the cytoplasm to the nucleus, where IE viral RNA synthesis begins using host RNA polymerases (Chinchar et al., 2009). As IE proteins produce transcription transactivators, they set the stage for transcription of DE genes (Chinchar et al., 2009). Subsequently, the viral material in the nucleus begins first stage DNA synthesis of genome-sized molecules using viral DNA polymerase (Chinchar et al., 2009). This viral DNA is then transported to the cytoplasm where concatameric (multiple repeats) structures

are produced and methylated by a virus-encoded cytosine DNA methyltransferase (Chinchar et al., 2009). This DNA methylation is critical to viral proliferation by preventing degradation due to host mechanisms (Chinchar et al., 2009). While the exact mechanism for this is unclear, some work suggests that methylation blocks toll-like receptor 9 (TLR) (Chinchar et al., 2009). This is crucial as unmethylated DNA will bind to TLR-9 and may induce inflammatory responses (similar to herpes) that inhibit viral spread (Chinchar et al., 2009). Furthermore, treatment with an inhibitor of DNA methylation (5-azacytidine) reduces virus production 100 fold (Chinchar et al., 2009).

Virions are assembled within the cytoplasm of the infected cell following late viral RNA and protein synthesis (Chinchar et al., 2009). Late viral proteins are thought to encode structural elements, including the major capsid protein (a major gene segment used in PCR identification of FV3) (Zhao et al., 2007; Chinchar et al., 2009). Inhibition of MCP protein transcription corresponds to a reduction in viral titers (Sample et al., 2006). Mechanisms of virion assembly are poorly known, but packaging of the viral DNA results in a terminally redundant and circularly permuted virion (Goohra and Mutri, 1982; Chinchar et al., 2009). This leads to a nonenveloped product that can be seen with electron microscopy in crystalline arrays (Chinchar et al., 2009). Some virions become enveloped as they bud from the plasma membrane; however, the majority of virions are released upon lysis of the infected cell (Chinchar et al., 2009).

There is a marked reduction in host DNA, RNA, and protein synthesis during FV3 infection (Chinchar et al., 2009). Proposed mechanisms that lead to cell death have included both necrosis and apoptosis, although recent evidence suggests that apoptosis is the predominant mechanism (Chinchar et al., 2003; Chinchar et al., 2009). This mechanism occurs in a host cell within 6-9 hours post-infection, as seen by DNA fragmentation, chromatin condensation, and

membrane reversal (Chinchar et al., 2009). With this virus, apoptosis can be triggered by a live virion or a heat and UV inactivated virus, which suggests viral protein is part of the driving mechanism (Chinchar et al., 2009). In soft-shelled turtle iridovirus (a ranavirus), it was observed that a mitochondria-mediated pathway was likely involved in the apoptotic pathway through caspase-3 activity (Huang et al., 2011).

Diagnosis

Several methods have been proposed for the diagnosis of ranaviruses, including histopathology, PCR, virus isolation, ELISA, electron microscopy, restriction fragment length polymorphism (RFLP), and cytology (Hyatt et al., 2000; Gray et al., 2009a; Miller et al., 2011).

Conventional PCR against the MCP is commonly used to identify infections, but is limited by the fact that it only allows detection and doesn't confirm active infection. Diagnosis is most successful on fresh or frozen tissues, but a method for successful recovery from formalin fixed tissues exists (Kattenbelt et al., 2000). Evaluation of ante-mortem (toe clip) vs. post-mortem (liver) samples in anurans was evaluated for detection of FV3 (St-Amour and Lesbarreres, 2007). The study found that 88% of samples that were positive in liver samples were also positive in toe clips, leading the authors to conclude that both methods are effective for detection (St-Amour and Lesbarreres, 2007). The specificity and sensitivity of PCR was also evaluated comparing post-mortem (necropsy) and ante-mortem (tail clips) in salamanders (Greer and Collins, 2007). The study demonstrated that tail clips underestimate prevalence, although its agreement increases as time after exposure increases (Greer and Collins, 2007). In reptiles, necropsy tissues are most commonly tested, while antemortem sampling has not been evaluated. Studies have reported the use whole blood and oral or cloacal swabs in red-eared slider turtles

and box turtles, but those results have not been validated against necropsy samples (Johnson et al., 2008; Allender et al., 2011). Future studies should evaluate the specificity and sensitivity of antemortem diagnostic sampling techniques.

Quantitative PCR (qPCR) has been developed to detect lower amounts of genetic material than conventional PCR, including several ranaviruses. A qPCR assay was developed for a 62-bp segment of the major capsid protein (MCP) of LBMV in largemouth bass during an experimental transmission (Getchell et al., 2007). LBMV persists in fish populations, and this assay was useful for detecting the subclinical state (Getchell et al., 2007). In a qPCR study for white sturgeon iridovirus, sensitivity was compared to histopathology (Kwak et al., 2006). The study found that the sensitivity of the qPCR assay was 98%, while histopathology was 64%, confirming that qPCR is a highly sensitive assay (Kwak et al., 2006). Quantitative PCR has been developed with primers targeting a segment of the ranavirus DNA polymerase gene (Holopainen et al., 2011). This assay was validated in cell culture for several ranaviruses and was found to be effective in detecting virus in fish tissues experimentally infected with EHNV (Holopainen et al., 2011). A similar diagnostic assay for reptiles is lacking.

A loop-mediated isothermal PCR assay (LAMP) was developed for detection of a segment of the TK gene in STIV (Liu et al., 2011). LAMP has the advantages of requiring simple equipment and having a rapid turnaround for results (Liu et al., 2011). When compared to qPCR in the same study, LAMP was shown to be 100 times more sensitive and the detection limit was 20 copies/µl (Liu et al. 2011). This has not been used in North American species, including reptiles, but may necessitate further investigation.

Several molecular methods have been designed to differentiate viral isolates. A conventional PCR was developed to differentiate EHNV and BIV with primers targeting an open

reading frame (Gould et al., 1995), and later combined with restriction endonuclease patterns to differentiate EHNV, BIV, Warmena virus, and FV3 (Marsh et al., 2002). To differentiate between closely related ranaviruses, three qPCR assays directed against the MCP, DNA polymerase, and neurofilament H1-like protein gene were developed (Holopainen et al., 2009). These assays were able to distinguish between closely related phylogeny of 11 isolates, including FV3, EHNV, and BIV, among others (Holopainen et al., 2009). Another qPCR assay was developed with the aim of differentiating EHNV, BIV, European sheatfish virus, and European catfish virus (Pallister et al., 2007), and while the assay was found to be sensitive for the detection of ranaviruses, it needs further development to be able to differentiate these viruses (Pallister et al., 2007).

Immunohistochemistry is useful in quickly confirming the presence of a pathogen in formalin fixed tissue. An immunohistochemical (IHC) method has been developed using rabbit antiserum against purified virus to definitively demonstrate systemic ranavirus infection in several tissues from fish, amphibians, and reptiles (Balseiro et al., 2009; Cinkova et al., 2010). Immunohistochemistry also has been developed in the UK that is capable of detecting ranavirus in lymphocytes, fibrocytes, and melanomacrophages, among other tissues from amphibians (Cunningham et al., 2008). IHC can be an invaluable diagnostic tool when working on an outbreak, and has been used in Australia for this purpose (Hyatt et al., 2002). However, IHC can only be applied to biopsy or necropsy tissues, which are an invasive method that is not a preferred method for antemortem diagnosis.

Serologic assays have been developed to detect antibodies against iridoviruses in fish, amphibians, and reptiles (Kim et al., 2007; Johnson et al., 2010; Zhang et al. 2010). Antibodies in cane toads (*Bufo marinus*) were detected using a newly-developed ELISA against purified

BIV and EHNV (Whittington and Speare, 1996; Whittington et al., 1997). The assay demonstrated stronger reactivity with BIV than EHNV in the cane toads, which was not surprising because BIV is an amphibian pathogen and EHNV is a piscine pathogen (Whittington et al., 1997). In a separate study, the additional step of pre-absorption of the toad sera with a monoclonal antibody against an EHNV protein improved the specificity of the ELISA, which the authors concluded made the assay a useful screening tool for toads (Zupanovic et al., 1998a). In chelonians, FV3 antigen and an anti-desert tortoise IgY were used in a secondary ELISA (Johnson et al., 2010). This assay demonstrated a high coefficient of variation (>15% in some replicates) but was used for evaluation of gopher tortoises and box turtles with low prevalence (Johnson et al., 2010), however the high coefficient of variation may have led to false positives.

While the previous diagnostic tests are specific to characterizing ranaviruses, additional diagnostic methods that are less specific should also be considered when attempting to determine the presence or absence of these viruses. Clinical pathology is commonly used in clinical and fieldwork to characterize the health status of an animal. While not specific, it can be used with other diagnostic methods in a parallel testing method to increase the sensitivities of the assays. Flow cytometry has been developed using bromodeoxyuridine to assess lymphocyte proliferation response (Morales et al., 2007), which may be helpful when evaluating a host response to a viral infection. Viruses can induce lymphopenia or lymphocytosis, depending on the virus; this has not been characterized with amphibians or reptiles and ranaviruses. Complete blood counts (CBC) may also prove valuable by providing insight into whether this group of viruses can induce inflammatory responses or leukopenias in these animals. A great benefit of these types of diagnostic methods compared to the others is that the samples can be screened quickly.

Pathogenesis

Many different factors have been shown to affect the pathogenesis of ranaviruses in fish, amphibians, and reptiles, including species susceptibility, environmental factors, and host immune response (Hoverman et al., 2010). However, there remains much we don't know about the pathogenesis of this group of viruses in these animals, especially reptiles. The following is a review of what has been done. Several ranavirus transmission studies have been performed in urodelans. Koch's postulates were originally satisfied for ATV in tiger salamanders using an isolate that was responsible for an epizootic in 1995 (Jancovich et al., 1997). Intracoelomic inoculation of ATV in salamanders led to mortality within 13 days (Bollinger et al., 1999). Lesions in affected salamanders included necrosis of several tissues, including: liver, spleen, skin, kidney, and hematopoietic tissues (Bollinger et al., 1999). ATV also has been shown to be transmissible by direct interactions (bumping, biting, cannibalism, necrophagy) and indirectly via water and fomites (Brunner et al., 2007; Gray et al., 2009; Hoverman et al., 2010). Specifically, larval amphibians are infective soon after exposure and transmission increases with time (Brunner et al., 2007).

Ranavirus transmission studies have also been done with anurans. A ranavirus isolated from an outbreak in free-ranging frogs from the UK induced death following systemic hemorrhages in inoculated frogs within 6 to 8 days post-infection (Cunningham et al., 2007a). Furthermore, a hemorrhagic syndrome seen in the UK frogs was shown to be more likely to result in disease compared with those animals demonstrating ulcerative signs alone (Cunningham et al., 2007b). These findings suggest that there might be tissue tropism for some viral strains that lead to differences in disease development (Cunningham et al., 2007b).

Transmission studies in reptiles, and more specifically turtles, are limited. Attempts to infect adult red-eared slider turtles using an oral inoculum of FV3 was unsuccessful; however, direct intramuscular injection of the virus was found to be effective at generating an infection model in this species (Johnson et al., 2007). To date, no attempts to infect turtles using water-borne or fomite based methods have been published, and while these techniques have been shown to be successful in infecting amphibians, there are clear anatomic and physiologic differences between these groups related to their integuments and how they absorb materials from the environment.

The effects of inoculum dose and host factors have been investigated for ATV in salamanders (Brunner et al., 2005). The study demonstrated that the time to the onset of clinical signs was correlated with inoculum dose, life history stage (juvenile), and genetic background (Brunner et al., 2005). In a follow-up study using a factorial design, ATV was experimentally transmitted to several familial lines of tiger salamanders) (Brunner et al., 2009). Virulence was highly correlated with viral strain and not amphibian lineage (Brunner et al., 2009). Similarly, a North American FV3 isolate was inoculated in Italian agile frogs (*Rana latastei*) and demonstrated a dose dependent survival and that cannibalism increased mortality rates (Pearman et al., 2004).

In Canada, FV3-like isolates from salamanders were used to experimentally infect wood frogs (*Rana sylvatica*); infection was found to be dose dependent (Duffus et al., 2008). The study went on to hypothesize that horizontal transmission through infected water was the most likely route of transmission for amphibians (Duffus et al., 2008). However, another transmission study in wood frogs demonstrated that scavenging on infected tadpoles was highly lethal (Harp and Petranka, 2008). Another experiment in the same species did not demonstrate increased mortality

(compared to controls) from infected water and sediment, but PCR was positive in those pools indicating possible sublethal infections (Harp and Petranka, 2008). The mixed results encountered in these studies should serve to reinforce the complexity of the epidemiology of ranaviruses in amphibians.

Attempts to definitely identify the vertebrate and invertebrate reservoir hosts for FV3 have been lacking. Much of this is because there have been limited field studies attempting to define the potential range of hosts. Attempts to evaluate different vertebrates using experimental models have been attempted with mixed results. Experimental FV3-like infections in black bullhead catfish (*Ameiurus melas*) failed to induce disease, but the virus could be re-isolated from euthanized fish at the conclusion of the study (Gobbo et al., 2010). However, the same experiment failed to re-isolate FV3-like DNA from surviving fish, suggesting this species is not likely to serve as a reservoir species (Gobbo et al, 2010). Experimental transmission in pike (*Esox lucius*) with FV3-like infections failed to cause significant mortality, but the virus was successfully re-isolated after infection; this case suggests that this species may serve as a subclinical carrier (Bang Jensen et al., 2009). In amphibian populations, proposed reservoir species include those that develop over one year (American bullfrog), have neotenic development (tiger salamanders), or have aquatic adult life stages (red-spotted newt, *Notophthalmus viridescens*; black-bellied salamander, *Desmognathus quadramaculatus*) (Gray et al., 2009). A PCR survey in Ontario, Canada, of both frogs and salamanders detected FV3-like virus in several species of both Orders (Duffus et al., 2008). They demonstrated that caudate salamanders are likely both the reservoir and the hosts of FV3 infections based on prevalence and viral load (Duffus et al., 2009). While in Brazil, South America, bullfrogs have been suggested as a reservoir species following an outbreak at an amphibian farm (Mazzoni et al.,

2009). In adult *Xenopus laevis*, persistence of viral DNA was detected in the kidneys, which could be activated when animals are immunosuppressed (Robert et al., 2007). Furthermore, peritoneal macrophages were also identified as a site of viral persistence and further support the likelihood that this species can serve as a viral reservoir (Robert et al., 2007). Because amphibians undergo a metamorphosis, it is possible that juveniles infected during their larval development could serve as potential reservoirs if they survive into their later adult life stage (Brunner et al., 2004).

The effect of ranavirus on life history traits such as survival, growth rates, and developmental stage was evaluated in the Northern leopard frogs (Echaubard et al., 2010). Experimental inoculation showed that increased animal density lead to a higher mortality rate, shorter time to death, longer developmental period, and lower growth rates (Echaubard et al., 2010). The authors speculated that individuals divert energetic resources away from the immune response to cope with increased environmental stress associated with high densities (Echaubard et al., 2010). Based on this theory, the emergence of ranavirus into naïve populations suffering from climate change, human encroachment, and other outside pressures could prove catastrophic.

The effect of temperature on the pathogenesis of iridoviruses has been shown to be important. In the rock bream fish (*Oplegnathus fasciatus*), red sea bream iridovirus induced high mortality at 18°, 21°, and 25°C, but not at 13°C (Jun et al., 2009), although after the 13°C temperature group was exposed to 25°C mortality reached 100% (Jun et al., 2009). In perch (*Perca fluviatilis*) and trout (*Oncorhynchus mykiss*) exposed to EHNV, mortality was greatest at higher temperatures (19°- 21°C), and was lowest or delayed at low temperatures (Whittington and Reddacliff, 1995). In the study, trout failed to develop clinical signs, but it was still possible to isolate the virus from the fish. The authors concluded that environmental persistence might

have played a role in the findings (Whittington and Reddacliff, 1995). In tiger salamanders experimentally inoculated with ATV, animals showed high mortality at 10°C and 18°C, while lower mortalities were noted at 26°C (Rojas et al., 2005). Furthermore, viral loads were highest in the animals kept at 10°C and lowest in the animals kept at 26°C (Rojas et al., 2005). While FV3 growth is inhibited at temperatures above 32°C, the effects of other ranaviruses in other species clearly indicate either virus or species-specific differences in pathogenesis, and further studies are needed in several species to better characterize the role of temperature in development of disease.

Disease susceptibility in amphibians, including to ranaviruses, has been linked to environmental contamination (Forson et al., 2006; Gray et al., 2007; Greer and Collins, 2008; Hayes et al., 2010). Atrazine exposure has been found to reduce peripheral leukocyte response in amphibians, and not surprisingly increase the susceptibility of larvae to ATV infection (Forson et al., 2006; Kerby et al., 2009). Chlorpyrifos (an organophosphate insecticide) has also been found to increase tiger salamander susceptibility to ATV (Kerby et al., 2009). While in some cases the source or type of contaminant is known, in others it is not. In these cases the contamination is insidious and often tied to some behavior. For example, amphibians with access to cattle farms (and likely environmental contamination) were three times more likely to be FV3 positive than controls (Gray et al., 2007). Tiger salamanders in Arizona were also more likely to be infected with ATV in ponds that had cattle access (Greer and Collins, 2008). Conversely, deltamethrin (a pyrethroid ester insecticide) was found to have very little immunosuppressive effect at environmentally relevant concentrations and not likely to lead to increased iridovirus infections (Froese et al., 2009). To date, the role of organic and inorganic contaminants on the susceptibility of reptiles to ranaviruses has not been evaluated, but merits investigation.

Besides toxicants in the environment, other anthropogenic factors have been shown to play a role in disease transmission in amphibians. The global trade in amphibians is a significant factor in disease epidemiology and threatens conservation; the concern for this has grown so large that it has received listing status by the Office International des Epizooties (OIE) to ensure the safety of international trade (Schloegel et al., 2009; Schloegel et al., 2010). It was suggested that human transport of bait salamanders from the Midwest USA to Arizona and Colorado was responsible for the introduction of ATV to these otherwise naïve environments (Jancovich et al., 2004; Jancovich et al., 2005; Picco and Collins, 2008). The authors concluded that because of the limited genetic variation seen in ranaviruses in North American salamanders, human movement is a plausible mechanism for its spread (Jancovich et al., 2005; Picco and Collins, 2008). Furthermore, in a survey of fisherman in the western USA, bait salamanders were commonly released into local waters, which the authors concluded could serve as a significant threat to releasing ranavirus into native populations (Picco and Collins, 2008). More importantly, these salamander isolates are able to infect but not kill fish in the environment, potentially leading to viral persistence and the spread of infections in the face of amphibian outbreaks (Picco et al., 2010). In a cross-sectional study evaluating *Rana clamitans*, the prevalence of ranavirus was influenced by industrial activity, degree of human influence, and distance to human habitation (St-Amour et al., 2008). It is clear from these studies that humans have both directly and indirectly increased ranaviral disease in amphibians, and therefore, it is reasonable to consider that these or other anthropogenic factors will occur in reptiles.

Innate or natural immunity of amphibians may play an important role in protecting susceptible species. Anti-microbial peptides (AMP), which are normal components of the mucous layer on amphibian skin, have been shown to significantly reduce viral infectivity

through inactivation of the virus (Chinchar et al., 2001; Chinchar et al., 2004; Rollins-Smith, 2009). However, when comparing channel catfish virus to FV3, FV3 were less sensitive to the effects of the anti-microbial peptides (Chinchar et al., 2004). This was supported by the suggestion that cell culture isolates (which have no AMP) were more likely to induce disease than infected amphibian skin itself (which have AMP) (Cunningham et al., 2007b). The role of peritoneal leukocytes has recently been evaluated as a critical innate defense mechanism in *X. laevis* (Morales et al., 2010). An increase in peritoneal leukocytes (1 day), natural killer cells (3 day), and T-cell response (6 day) are observed after infection (Morales et al., 2010). In addition, proinflammatory cytokine genes including interleukin-1β, tumor necrosis factor, and arginase 1 are upregulated in response to FV3 infection (Morales et al., 2010). When evaluating the role of these leukocytes, it was found that peritoneal leukocyte response was weaker than the response in the kidney, which the authors concluded was the main site of infection (Morales et al., 2010). This may be helpful in identifying sites of viral persistence in subclinical or reservoir hosts. Similar immune responses have not been discovered in reptiles

Adaptive immune responses have been shown to induce a CD8 T-cell response and IgY production in adult *X. laevis*, which shows protection upon re-infection with FV3 (Gantress et al., 2003; Robert, 2010). Anti-FV3 antibodies in adult frogs are not detected using ELISA after a primary infection, but are robust within 10 days to 8 weeks after secondary infection (Maniero et al., 2006; Robert, 2010). However, mRNA is active in B-cells during the primary infection, indicating that despite there not being detectable IgY, the antibody response is active (Robert, 2010). In an experimental ranavirus transmission study using red-eared slider turtles, ELISA positive results were not consistently observed within 30 days, nor were they observed reliably in Australian reptiles after experimental exposure (Johnson et al., 2007; Ariel, 1997). It is possible

that a similar mechanism in reptiles exists compared to amphibians, in which antibody development is robust only after a secondary infection. It was demonstrated in *X. laevis* that CD8 T-cells were upregulated within 6 days post-infection after primary infection and sooner after secondary infection (Morales et al., 2007). The authors concluded that CD8 T-cells provided *in vivo* protection against ranavirus in *X. laevis* (Morales et al., 2007). Larval amphibians infected with ranavirus have been found to experience greater mortalities than adults; these animals were also found to be deficient in the expression of MHC class I (Gantress et al., 2003; Robert, 2010). However, the authors caution that the lack of MHC class I molecules is not the only mechanism involved in disease development in larvae, as IgY conversion is not efficient in larval amphibians either and may play a role as well (Gantress et al., 2003). The adaptive immune response in reptiles is temperature dependent and future studies should evaluate the response to FV3 under various environmental conditions.

Physiologic responses of the hypothalamus-pituitary-interrenal axis (HPI) to infection have been evaluated in wood frogs and can influence pathogenesis (Warne et al., 2011). The authors determined that FV3-infected tadpoles had significantly higher corticosterone, rapid developmental changes, and a decrease in weight compared to controls (Warne et al., 2011). The authors concluded that while corticosterone usually enhances immunity, it accelerates metamorphosis that may be maladaptive in juveniles (Warne et al., 2011). Therefore, the epidemiology of FV3 infections in larval amphibians may be due to the energetic trade-offs between metamorphosis and response to infection and might favor those individuals that are in better body condition (Warne et al., 2011).

In conclusion, many factors affect the immune response and pathogenesis that still need to be elucidated in amphibians and reptiles. However, it is apparent that environmental

temperature plays a role in the development of disease either through virus-specific factors (e.g. growth not supported above 32°C, possible tissue specificity) or host factors (e.g. immune response is more robust at higher temperatures in reptiles, habitat preference [aquatic or terrestrial], age [juveniles have less developed immune system to fight infections than adults]). Furthermore, the presence of FV3 in reservoir species or other factors (pond substrate) need to be determined for reptiles.

Treatment

Therapeutic interventions in free-ranging settings are often impractical for combatting disease outbreaks. However, in captive animals or in those situations where an endangered group of free-ranging animals are brought into captivity, an appropriate treatment protocol needs to be established. Therapeutic protocols to many viral diseases in reptiles have not been established. However, several texts have recommended the use of anti-viral medicine (acyclovir) in treatment of herpesvirus infections in tortoises.

Acyclovir is a guanine analogue antiviral drug (Elion, 1993). It is active against herpesviruses due to the presence of the thymidine kinase (TK) enzyme, which rapidly activates acyclovir to the monophosphate form (Beutner, 1995). Once phosphorylated three times, the antiviral competes with deoxyguanosine triphosphate (dGTP) for the viral DNA polymerase. Acyclovir uptake has been shown to be enhanced in herpesvirus-infected cells, with a 10- to 30-fold greater affinity in infected cells than uninfected cells (Beutner, 1995). Once incorporated into the viral genome, relatively low levels of the drug are needed to achieve viral inhibition and adequate intracellular concentrations can be maintained for several hours (Beutner, 1995). Valcyclovir, an esterified version of acyclovir, is rapidly converted to acyclovir after absorption

and has greater oral bioavailability than acyclovir (Garre et al., 2008). In humans, the oral bioavailability of valcyclovir is 3-5 times greater than acyclovir, while in horses and cats it is 8 times and 2-3 times more bioavailable than oral acyclovir itself, respectively (Garre et al., 2007; Nasisse et al., 1997).

Several isolates of iridoviruses have been shown to have thymidine kinase genes or functional TK enzymes (Coupar et al., 2005; Jakob et al., 2001; Scholz et al., 1988; Tsai et al., 2005). Inhibition of this gene has been shown to slow viral replication (Coupar et al., 2005). When genetically compared, iridoviruses and herpesviruses appeared to have more closely related mitochondrial TKs (Coupar et al., 2005). This suggests that if iridovirus TK genes are similar to herpesvirus TK genes, it is plausible that they too could phosphorylate and activate acyclovir. The activity of valcyclovir has been shown to be entirely due to the conversion to acyclovir, but with higher bioavailability (Beutner, 1995).

The half-life of acyclovir after a single oral dose varies in humans (3.1 h), cats (3.1 h), and horses (5.05 h) given oral valcyclovir and marginated tortoises (*Testudo marginata*) (8.8 h) given oral acyclovir (Gaio et al., 2007; Owens et al., 1996; Garre et al., 2007; Beutner, 1995). The results demonstrate a slower elimination in turtles, which could reduce dosing intervals.

The bioavailability of acyclovir in humans, horses and cats is 20%, 3-8%, and 15-21%, respectively, and it decreases with increasing doses (Beutner, 1995; Wilkins et al., 2005; Owens et al., 1996; Nasisse et al., 1997).

Acyclovir has been proposed for treatment of herpesvirus in several species, and for iridovirus in chelonians (Beutner, 1995; Gaio et al., 2007 DeVoe et al., 2004; Funk and Diethelm, 2006; Marschang et al., 1997). For herpesvirus in humans and horses, inhibitory concentrations need to be maintained above 0.45 μg/ml and 3 μg/ml, respectively (Gaio et al.,

2007). However, in vitro concentrations that completely inhibit tortoise herpesvirus were above 50 μg/ml (Marschang et al., 1997). Additionally, in vitro studies against an iridovirus using acyclovir indicated only a dose-dependent partial inhibition at 25 μg/ml (Johnson, 2006). There have been no *in vivo* studies evaluating efficacy or inhibitory concentrations in chelonians against either virus. Future studies should evaluate *in vivo* efficacy against iridoviruses to determine if higher concentrations of drug are reached in infected cells, as has been demonstrated with herpesviruses in other species.

Toxicity of therapeutic use using these anti-virals is unknown in turtles. In cats, nephrotoxicity and bone marrow suppression were seen with repeated dosing of acyclovir and valacyclovir (Owens et al., 1996). If studies evaluating these drugs are pursued in reptiles, animals should be monitored closely for these potential side effects.

Besides pharmacologic treatment, other mechanisms to control FV3 include disinfection. One study investigated the effects of chlorhexidine, sodium hypochlorite, potassium peroxymonosulfate, and potassium permanganate on ranavirus (Bryan et al., 2009). Chlorhexidine at 0.75%, sodium hypochlorite at 3%, and potassium peroxymonosulfate at 1% were all effective at inactivating ranavirus after a 1 minute exposure (Bryan et al., 2009), while, potassium permanganate (100% concentration) after a 60 minute exposure, chlorhexidine at 0.25% and sodium hypochlorite <3% were found to be ineffective (Bryan et al., 2009).

Field studies in areas where infectious diseases are present risk human spread of ranaviruses between field sites. Hygiene methods have been established for amphibians to reduce this potential risk: 1) low stringency – clean and dry equipment and clothing; 2) moderate stringency – disinfect equipment and vehicle; and 3) high stringency – site-dedicated or disposable equipment or thorough cleaning and vehicle is washed and disinfected (Phillott et al.,

2010). Furthermore, the authors have established a set of easy to use criteria to determine the risk based on prior activity, proposed activity, remoteness of site, presence of pathogen at site, and population status of all species at site (Phillott et al., 2010).

Table 2.1. Species and locations ranaviruses have been isolated from free-ranging amphibians and reptiles.

Class	Continent	Country	State/Province	Family	Virus	References
Amphibia	North America	USA	New Hampshire Colorado North Dakota Minnesota Maine Utah Idaho Tennessee Massachusetts Wyoming North Carolina Arizona Texas Virginia	*Ranidae* *Ambystomatidae* *Hylidae* *Bufonidae* *Plethodontidae* *Cryptobranchidae* *Scaphiopodidae*	Unclassified iridovirus FV3 ATV	Jancovich et al., 1997 Green, 2002 Docherty et al., 2003 Converse and Green, 2005 Gray et al., 2007 Gray et al., 2009a Glenney et al., 2010 Torrence et al., 2010 Davidson and Chambers, 2011 Miller et al., 2011
		Canada	Saskatchewan Ontario Quebec	*Ambystomatidae* *Ranidae* *Hylidae* *Salamandridae*	ATV	Bollinger et al., 1999 Greer et al., 2005 Duffus et al., 2008 St-Amour et al., 2008 Schock et al., 2010a Echaubard et al., 2010 Schock et al., 2010b Paetow et al., 2011

Table 2.1, continued.

	Continent	Country	State/Province	Family	Virus	References
Amphibia, cont.	South America	Venezuela Brazil Uruguay Argentina		*Bufonidae* *Ranidae* *Leptodactylidae*	Unclassified iridovirus FV3	Zupanovic et al., 1998c Galli et al., 2006 Fox et al., 2006
	Europe	United Kingdom Croatia Spain Belgium Netherlands Israel Portugal		*Ranidae* *Discoglossidae* *Salamandridae* *Bufonidae* *Alytidae*	Unclassified ranavirus FV3	Fijan et al., 1991 Drury et al., 1993 Cunningham et al., 1996 Hyatt et al., 2000 Pasmans et al., 2008 Alves de Matos et al., 2008 Balseiro et al., 2009 Ariel et al., 2009 Duffus et al., 2010 Balseiro et al., 2010 Hyatt et al., 2010
	Australia	Australia		*Myobatrachidae*	Unclassified ranavirus	Speare and Smith, 1992
	Asia	China Japan		*Ranidae*	FV3	Xu et al., 2010 Une et al., 2009

Table 2.1, continued.

	Continent	Country	State/Province	Family	Virus	References
Reptilia	North America	US	Florida Georgia Florida Pennsylvania New York Texas Tennessee North Carolina Kentucky	*Testudinidae* *Emydidae*	FV3 or FV3-like	Westhouse et al., 1996 Johnson et al., 2006 Ruder et al., 2010 Allender et al., 2011
	Asia	China		*Trionychidae*	STIV	Chen et al., 1999

Table 2.2. Reports of non-erythrocytic iridoviruses in captive reptiles.

Continent	**Country**	**Family**	**Virus**	**Reference**
North America	USA	*Testudinidae*	FV3	Johnson et al., 2006
		Emydidae	FV3-like	DeVoe et al., 2004
Europe		*Agamidae*	Chilo-iridescent virus	Just et al., 2001
		Chameleonidae	Chilo-iridescent virus	Just et al., 2001
	Germany	*Gekkonidae*	FV3	Marschang et al., 2005
	Switzerland	*Testudinidae*	Unclassified ranavirus	Benetka et al., 2007
	Germany	*Testudinidae*	FV3-like	Marschang et al., 1999
	UK	*Chameleonidae*	Unclassified iridovirus	Drury et al., 2002
Australia		*Boidae*	BIV	Hyatt et al., 2002
Asia	China	*Trionychidae*	STIV	Chen et al., 1999

CHAPTER 3

DEVELOPMENT AND VALIDATION OF TAQMAN QUANTITATIVE PCR FOR DETECTION OF FROG VIRUS 3-LIKE VIRUS IN EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*)[1]

Objective: Develop a quantitative real-time PCR (qPCR) assay using a TaqMan probe-based assay derived from a highly conserved region of the major capsid protein of frog virus 3 (Family *Iridoviridae*, genera *Ranavirus*) and use the qPCR to identify FV3 or FV3-like infections in a collection of 31 eastern box turtle samples.

Design: Assay development for cross-sectional study

Animals: Thirty-one eastern box turtles (*Terrapene carolina carolina*)

Procedures: Standard curves were generated from a viral DNA segment cloned within a plasmid. Blood and oral swab samples from free-ranging box turtles were evaluated.

Results: The TaqMan assay detected viral DNA copies 1000 times lower than conventional PCR. The linear range for TaqMan was between 5.3×10^9 to 5.29×10^4 with an R^2 of 0.999 (slope = -3.3; 100% efficiency). The prevalence of ranavirus in blood was 3% (95% CI:0-9) and was 6% (95% CI: 0-14) in oral swab samples.

Conclusions and Clinical Relevance: Viral isolation and conventional PCR are the most common methods for diagnosis. In this study, a qPCR was developed that allows for a superior, rapid, sensitive, and quantitative method for detecting *Ranavirus* in box turtles. Thirty-one clinical

[1] Content of this chapter has been partially or fully submitted for publication as cited in bibliography as Allender et al., 2012a.

samples (whole blood and oral swabs) from box turtles were tested using this assay and the prevalence of the virus determined. This assay will be useful for early detection and disease monitoring. .

Introduction

Ranavirus is one of five genera from the family *Iridoviridae* and one of three genera reported to infect reptiles. They are large, icosohedral, DNA viruses that have emerged as a significant cause of mortality events in free-ranging herpetofauna throughout the world (Green et al., 2002; Johnson et al., 2008; Miller et al., 2011). *Ranavirus* has specifically been linked with numerous outbreaks occurring in the US (Green et al., 2002; Jancovich et al., 2003). Recently, ranaviral disease in amphibians was placed on the World Organization for Animal Health list of reportable diseases. Disease events in amphibians are often clustered into local epizootics, with significant impact on the local population.[5] These epizootics have been scattered across numerous habitats and landscapes in the US; however, disease predictability has not been successful. Furthermore, there have been increasingly more reports and cases in chelonians, and specifically box turtles, in the US (Allender et al., 2006; DeVoe et al., 2004; Johnson et al., 2008).

Diagnostic assays that are validated and optimized to detect the presence of pathogens in certain species are key to characterizing the disease and understanding disease ecology. Quantitative PCR (qPCR) has not been previously developed for detection of ranavirus in turtles. Its use would likely provide much greater sensitivity in detection of ranavirus as compared with conventional PCR, and additionally would allow the quantitation of virus levels within specific tissues. Identifying clinical samples with the highest viral load will allow for more efficient sampling methods that target those tissues and potentially direct therapy toward those sites. Additionally, the greater sensitivity of qPCR will allow for the detection of ranavirus in animals with lower titers of virus, as might be seen in early or subclinical infections. In studies investigating iridoviruses in fish, TaqMan real-time PCR was shown to be 100 times more

sensitive than conventional PCR, and crucial for the identification of subclinical disease states (Getchell et al., 2007; Pallister et al., 2007). Animals with subclinical infections may serve as important carriers or reservoirs for infectious disease; therefore, it is critical to develop assays capable of detecting the pathogen in these animals. To date, no such assay has been reported for ranavirus quantification in free-ranging or captive chelonians.

The purpose of this study was to develop and evaluate different diagnostic methods for characterizing an emerging pathogen, frog virus 3-like virus (FV3; genera *Ranavirus*, Family *Iridoviridae*) in box turtles. The hypotheses tested in this study were that a qPCR TaqMan based assay would be both sensitive and specific for characterizing FV3-like disease in box turtles. This is essential when considering the application of these assays to free-ranging populations and/or experimental models. Furthermore, it allows for evaluating potential climatic and environmental impacts on the disease, as well as treatment, and management options.

Materials and Methods

DNA extraction

Whole blood and an oral swab were collected from a positive eastern box turtle that presented to the University of Tennessee (Knoxville, Tennessee, USA) wildlife clinic in 2007. DNA was extracted following manufacturers instructions (QIAamp DNA blood mini kit, Qiagen, Valencia, CA). Concentrations and purity of DNA were determined using a spectrophotometer (Nanodrop spectrophotometer, Thermo Scientific, Wilmington, DE).

Conventional PCR, Sequencing, and Cloning

Conventional PCR was performed using primers targeting a portion of the major capsid protein (MCP) (Mao et al., 1997) Products were sequenced in both directions using the

University of Illinois Core DNA Sequencing Facility (Urbana, Illinois, USA) and compared to known sequences in GenBank using TBLASTX. For cloning, the PCR product (531 bp) from the MCP 4 primers (Mao et al., 1997) was then cloned in *E. coli* (TOPO TA Cloning® kit, Invitrogen, Carlsbad, CA). The cloning product was verified through sequencing in both directions. Plasmids were linearized with EcoR1, purified (QIAfilter plasmid Maxi kit, Qiagen, Valencia, CA), and quantified using spectrophotometry. Ten-fold serial dilutions of linearized plasmids were made from 10.0×10^{2} ng/µl to 10×10^{-8} ng/µl. Viral genome (DNA) copy number was calculated using the following formula:

$$\#\ \text{copies}/\mu\text{L} = \frac{(\text{ng DNA of plasmid + clone}/\ \mu\text{L})\ (6.022 \times 10^{23}\ \text{copies/mol})}{(\text{bp length})(1 \times 10^{9}\ \text{ng/g})\ (650\ \text{g/mol of bp})}$$

The final copy number for ten-fold serial dilutions ranged from 5.29×10^{9} to 5.29×10^{1} viral copies per reaction.

Real time qPCR Assay

A primer/probe assay for a TaqMan-MGB **(**TaqMan® primers, Applied Biosystems, Carslbad, CA**)** based qPCR assay were designed using a commercial software program (Primer Express®, Applied Biosystems, Carlsbad, CA) based on published sequences of the major capsid protein of FV3 (Mao et al., 1997). TaqMan assay was performed using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA), and probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein (MCP) of frog virus 3. Real-time qPCR assays were performed using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA) and data was analyzed using associated software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, CA). Each reaction contained 12.5 µl of 2x TaqMan Platinum PCR Supermix-UDG with ROX

(TaqMan Platinum PCR Supermix-UDG with ROX, Invitrogen, Carlsbad, CA), 1.25 µl TaqMan primer-probe, 2.5 µl turtle-derived FV3 dilution, and water to a final concentration of 25 µl. Cycling parameters were as follows: 1 cycle at 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds, and a final cycle of 72°C for 10 minutes.

Standard curve, Specificity, and Sensitivity

To determine the sensitivity, assays were performed in three technical repeats on dilutions of turtle-derived positive control plasmid of FV3 MCP DNA ($5.29x10^9$ - $5.29x10^1$ copies/rxn) within a single run. Standard curves were generated using the cycle threshold (Ct) values of the positive control plasmid dilutions. Intra-assay variation was determined for both assays by calculating the mean Ct values, standard deviations (SD), and coefficient of variations (CV) separately for each control plasmid DNA dilution. Efficiency curves of the dilutions were performed in uninfected cell culture lysates (spiked with plasmid dilutions), infected cell lysates, and turtle whole blood extracts from a positive sample.

Box turtle samples

Thirty-one eastern box turtles that were presented to the University of Tennessee College of Veterinary Medicine Wildlife clinic for treatment of either traumatic or infectious disease identified by Good Samaritans were sampled during March through October 2007. Whole blood and swabs of the oral cavity were collected as previously described (Allender et al., 2011). The University of Tennessee Institutional Animal Use and Care Committee approved all animal use (protocol 1864). Results for detection were compared to published conventional PCR results for the same samples (Allender et al., 2011).

Statistical Analysis

The quantity of ranavirus target DNA in infected whole blood and oral swabs was determined using a standard curve method. The copy number of the target DNA was determined from the standard curve generated with ten-fold dilutions of the positive control plasmid that contained the target sequence of the respective qPCR assay.

Copy numbers were tabulated and evaluated for normality using the Shapiro-Wilk test. Mean, median, standard deviation, 95% confidence interval, and 10-90% percentiles were determined for positive cases (copy number) for each assay. The Mann-Whitney U test was used to evaluate between assay differences. The prevalence of ranavirus was determined for each assay (categorical variable assigned; 1=positive, 0=negative). Exact 95% binomial confidence intervals were determined for all proportions. Level of agreement (kappa) was determined between both the real-time PCR assays and the conventional PCR previously reported based on prevalence. All statistical analysis was performed using statistical software (IBM SPSS Statistics 20, Chicago, IL).

Results

Conventional PCR was performed to evaluate the level of detection of turtle-derived FV3-like dilutions (Figure 3) (Mao et al., 1997) The TaqMan primer set was designed to detect a 54 bp length gene segments of a conserved portion of the MCP gene of FV3.

Serial ten-fold dilutions of positive control plasmids were assayed with our TaqMan assay and standard curves were generated based on Ct values (Figure 1). The linear range for TaqMan was between 5.29×10^9 to 5.29×10^4 with an R^2 of 0.999 (slope = -3.277) (Figure 1). Efficiency curves carried out on spiked-uninfected cell lysates (controlled for total DNA per

rxn), infected cell lysates, and turtle whole blood extracts performed equally well (data not shown). The amplification plots of TaqMan qPCR are presented in figure 2.

The intra- and inter-assay reproducibility was evaluated for the serial dilutions of the control plasmids (Table 2). The intra-assay CVs for TaqMan were between 0.04 – 0.24%. The inter-assay CVs were 0.14 – 0.3%, respectively. These results indicate high reproducibility between assays at all dilutions. The dynamic range for qPCR assays at which point the C_t value of the triplicates was from 5.29×10^9 to 5.29×10^1.

Box turtle samples

Twenty-nine blood samples and thirty oral swabs were collected routinely from turtles presented to the University of Tennessee from March through October 2007. Mean quantity of DNA recovered from blood samples measured through spectrophotometry was 726 ng/ul (95% CI: 550.38 – 902.05) and mean purity A260/280 was 1.85 (95%CI: 1.82 – 1.88). Median quantity of DNA recovered from swabs was 19.42 ng/µl (10-90%: 3.22 – 57.06) and A260/280 was 1.81 (10-90%: 1.20 – 2.05). Conventional PCR assays previously reported a 3% (n=1; 95% CI:0-9) prevalence rate for these samples (Allender et al., 2011). Quantitative PCR determined the presence of ranavirus in blood of the same individuals (prevalence = 3%; n=1; 95% CI:0-9). However, prevalence of ranavirus in the oral swab samples using qPCR identified the same individuals as conventional and an additional positive animal (prevalence = 6%; n=2; 95% CI: 0-14). Mean viral quantity in blood for the single animal that was positive using TaqMan was 1.72×10^8. The mean viral copy number in the swab sample from the same individual was 6.48×10^8. The number of viral copies in the other swab sample that was positive (no corresponding positive blood sample) was 4785. Level of agreement between was substantial between conventional PCR and TaqMan qPCR (kappa=0.651).

Discussion

Ranaviral disease is one of only two amphibian diseases listed as reportable to the World Organization for Animal Health. This is in part due to the mass mortality events that have affected amphibian populations worldwide (Miller et al., 2011). Despite its listing and severity of disease outbreaks, the epidemiology of this virus in amphibians, and hence reptiles, is not completely understood. Future and current epidemiologic surveys that determine the extent of disease and species range depend on diagnostic assays. The current study developed a quantitative PCR assay that allows detection of ranavirus in levels up to 1000 viral copies below conventional PCR based on dynamic range and levels of detection. The greater sensitivity provides the opportunity to potentially detect early, subclinical, or reservoir states of the disease, which are crucial factors, that are currently unknown, but needed to characterize the epidemiology of ranavirus.

The level of detection included a lower viral copy than the linear range for the assay. The linear range was more reliable for quantification of higher viral copies than lower viral copies. It is likely that primer concentrations need to be optimized for low viral copy numbers, resulting in a separate protocol based on viral copy. Future studies should lower primer concentrations in assays of with less than 5.29×10^4 viral copies to establish a linear range that is more reliable for detection and quantification of low viral copy numbers. In the absence of that optimization, the developed assay is reliable for absolute quantitation of ranaviral DNA, with greater than 5.29×10^4 viral copies and relative quantitation for the entire dynamic range. This might not biologically significant for this disease in this species, as viral quantity in infected animals is frequently above 5.29×10^4 viral copies (Allender, unpub. data).

TaqMan assays utilize a third selection step (probe), which provides for greater specificity not seen with other qPCR assays. The TaqMan assay is more stringent in requiring the hybridization of a probe to target sequence between the PCR primers. Its degradation during PCR amplification is responsible for emitting a fluorescent signal. This additional step provides for a higher specificity when testing clinical samples. The assay had highly reproducible results with intra- and inter-assay variability coefficient of variation of less than 5%. The primers were designed to be specific for a segment of the major capsid protein gene that previous studies have targeted with conventional PCR (Mao et al., 1997). It is highly conserved among various ranaviruses (Jancovich et al., 2010; Jancovich et al., 2005; Jancovich et al., 2003; Liu et al., 2007; Marsh et al., 2002).

DNA from twenty-nine blood samples and thirty oral swabs were screened for ranavirus. These samples were selected because they had previously been screened using conventional PCR (Allender et al., 2011). The results indicate that the TaqMan qPCR assay developed in this study was able to only identify a single additional positive oral swab sample, which was not identified using conventional PCR (Allender et al., 2011). This may signify that this population of turtles indeed had a low prevalence of FV3-like DNA (not infected), were not shedding ranavirus in the sample, or did not have enough viral copies to return a positive result. The parallel testing strategy used evaluating the two assays suggests that indeed the prevalence of infection was low in this population of box turtles. The pathogenesis of ranavirus in turtles is not known, and therefore it is possible that when a susceptible individual is exposed to the virus, it replicates quickly to a level above 529,000 viral copies (conventional PCR level of detection) or does not replicate well. This would explain the results of conventional PCR and qPCR being similar. Regardless of the viral copies present in a clinical animal (as represented by these samples), early

and subclinical detection that might detect the intermediate viral stage between exposure and high viral copy will provide a valuable tool for control, monitoring, and management of this disease. However, specific transmission studies are needed to determine whether a subclinical or carrier state even exists in chelonians. To further elucidate the sensitivity of this assay compared with conventional PCR, it will be important to apply it to a set of experimental samples where the prevalence of disease is high. As such, the quantitative assays reported here may be more useful in detecting and tracking the persistence and development of disease as once the animals present clinically they are likely overwhelmed with similar viral loads.

Concentrations of DNA, measured by spectrophotometry, were greater from extracted whole blood samples than oral swab samples. This is not surprising, as whole blood of reptiles contains nucleated red blood cells, which leads to significantly more DNA. Swab samples mostly contain epithelial cells in fewer numbers and therefore less DNA. This difference may not be diagnostically significant as viral DNA concentrations are not necessarily increased in whole blood samples, and furthermore the increase in host DNA may lead to reduced efficiency during PCR assays. Additionally, the assays are not based on a uniform amount of isolated material, therefore, blood should be normalized based on milliliter of blood. There were too few positive turtle samples to evaluate if higher DNA concentrations correlated with higher viral load, but future studies should investigate this correlation. Purity of DNA, determined by A260/A280 ratio, was within the ideal range (1.7 – 1.9) based on extraction guidelines for both whole blood and swab samples. However, the ratio was non-normally distributed for swab samples and the range was greater than whole blood samples. When the purity of the sample is outside the ideal range, it can lead to a decrease in assay efficiency or an increase in non-specific parameters and

inaccurate results. Therefore, purity should be evaluated prior to qPCR assay for all swab samples and samples outside the ideal range should be further purified before assaying.

The current study demonstrated that a TaqMan assay is reliable, specific, and sensitive for the detection of a gene segment of the MCP of frog virus 3. The TaqMan assay has low variability and was not likely to produce a non-specific product because of the additional probe step and therefore is the recommended assay for turtle ranavirus surveys. This assay can be used as a tool in the conservation of turtles by identifying emerging and ongoing outbreaks. This assay is more sensitive in detecting fewer viral copies than conventional PCR, thereby allowing for early, subclinical, or reservoir status detection if those states exist in turtles. As ranavirus continues to emerge as a major threat to reptiles and amphibians, it is critical that early and accurate identification of epidemics occur.

Table 3.1. Intra- and inter-assay variability of two SYBR green and one TaqMan qPCR assays detecting frog virus 3 major capsid protein.

	Intra-assay			Inter-assay		
Viral Copy	CT mean	CT SD	CV	CT mean	CT SD	CV
TaqMan						
5290000000	13.14	0.02	0.14%	13.14	0.03	0.19%
529000000	16.45	0.04	0.20%	16.45	0.04	0.23%
52900000	19.93	0.03	0.14%	19.94	0.03	0.14%
5290000	23.31	0.04	0.14%	23.30	0.04	0.16%
529000	26.34	0.07	0.23%	26.30	0.09	0.30%
52900	29.36	0.08	0.24%	29.33	0.07	0.22%
5290	30.16	0.02	0.04%	30.14	0.05	0.15%
529	31.24	0.05	0.14%	31.17	0.09	0.26%
52	31.80	0.01	0.03%	31.85	0.07	0.19%
NTC	37.94	1.05	2.26%	37.94	1.05	2.26%

Figure 3.1. Standard curve for a TaqMan probe-based primers obtained with tenfold serial dilutions from $5.29x10^9$ to $5.29x10^4$ FV3-like viral copies per reaction. The graph is plotted against a logarithmic concentration of the serial dilutions.

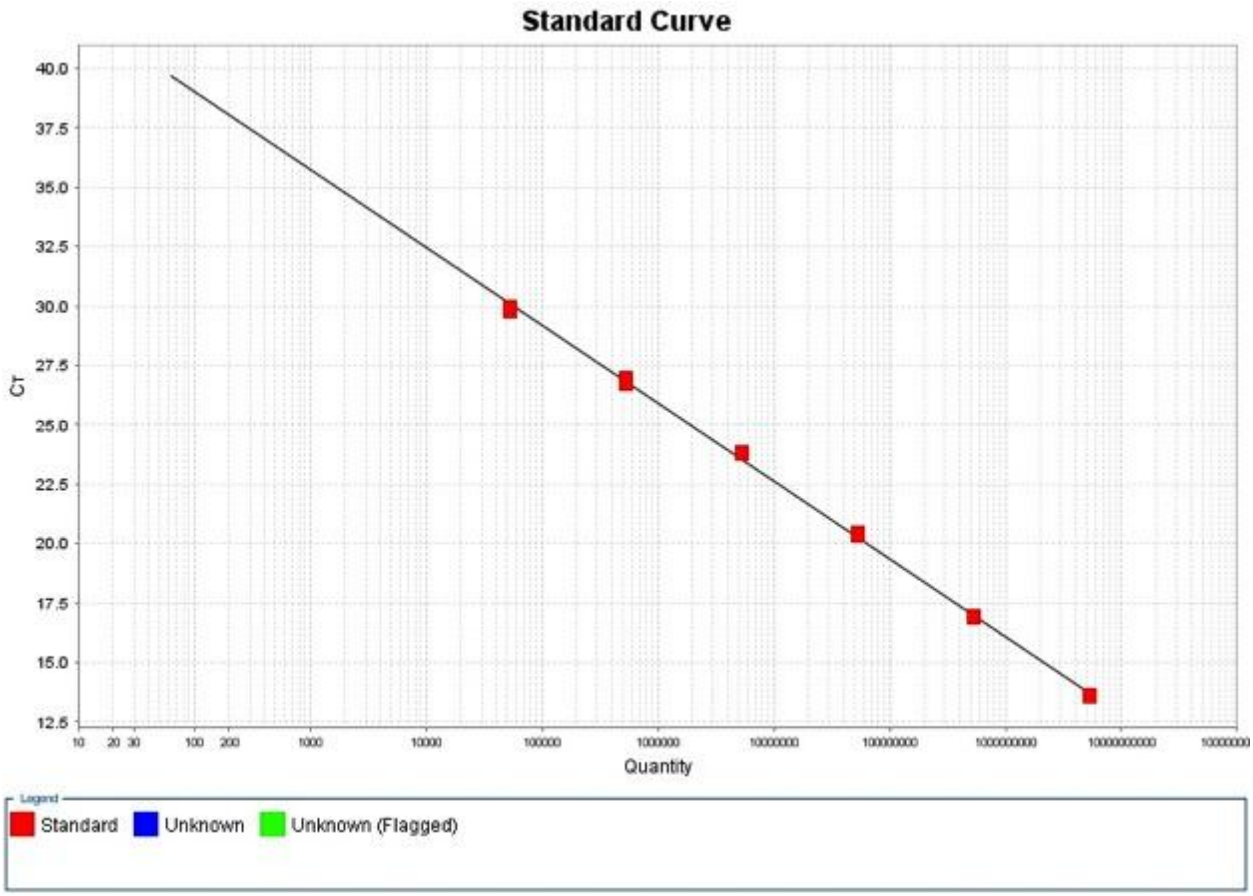


Figure 3.2. Amplification plots for the standard curve for TaqMan probe-based primers obtained with tenfold serial dilutions from $5.29x10^9$ to $5.29x10^4$ FV3-like viral copies per reaction plotted as the change in signal generated by each PCR amplicon (ΔRn) during each cycle.

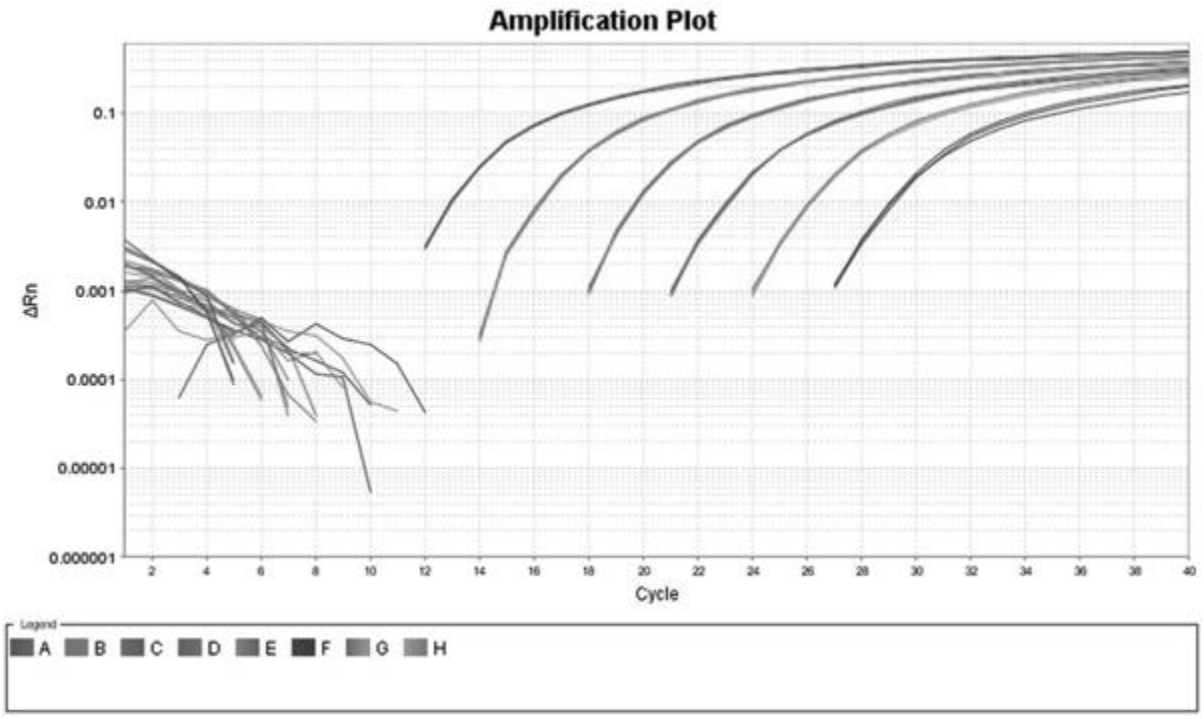
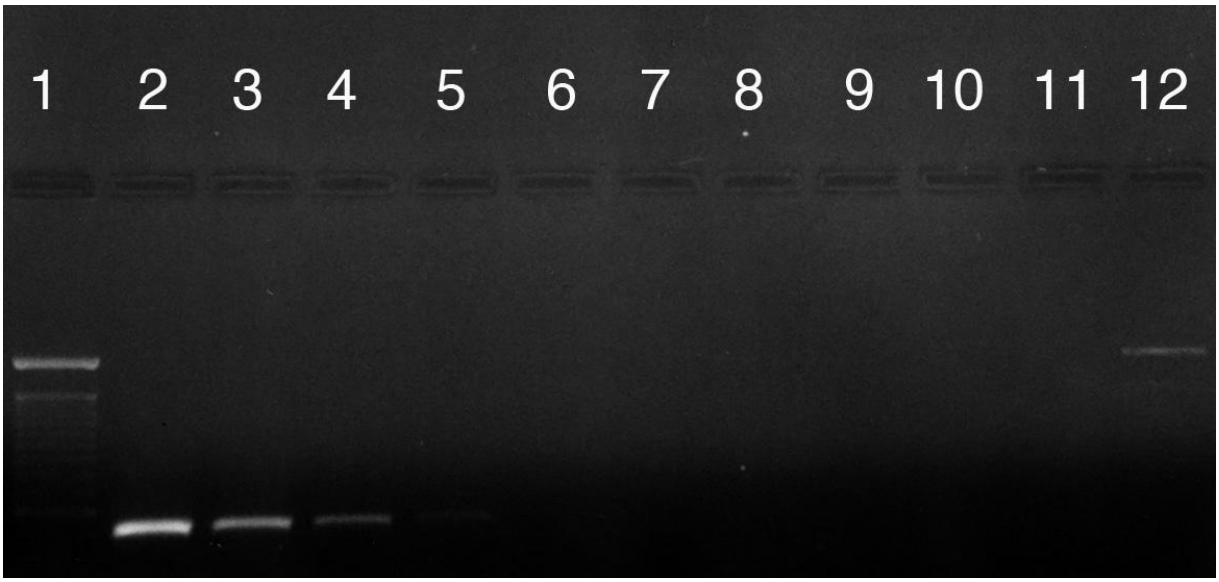


Figure 3.3. Gel electrophoresis of conventional PCR products using the MCP 4 primer targeting the major capsid protein of frog virus 3. Lane 1: 100 bp marker; Lane 2-11: Tenfold serial dilutions from $5.29x10^{8}$ to $5.29x10^{1}$ viral copies per reaction; Lane 12: 100 bp marker.



CHAPTER 4

PREVALENCE, CLINICAL SIGNS, AND NATURAL HISTORY CHARACTERISTICS IN EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*) WITH FROG VIRUS 3-LIKE VIRUS [2]

Abstract: Ranaviruses, specifically frog virus 3-like virus (FV3-like), have been associated with chelonian mortality events and may threaten biodiversity. To help characterize the disease ecology of FV3-like in chelonians, 606 free-ranging eastern box turtles (*Terrapene carolina carolina*) from either a free-living setting or those presented to rehabilitation centers in Tennessee, Virginia, North Carolina, Alabama, and Georgia were sampled from 2007 through 2011. Whole blood and swabs of the oral mucosa were collected from 458 adults (248 female, 199 male, 1 unknown sex) and 61 juveniles. Sex and age were unknown in 87 individuals. The prevalence of infection using quantitative PCR was 1.5% (n = 8; 95% CI: 0.8 – 2.9%). The prevalence in turtles presented to rehabilitation centers (n=7, 3.13%; 95% CI: 1.5 – 6.3%) than free-living turtles (n=1, 0.3 %; 95% CI: 0 – 1.8%). Four of the individuals were positive in whole blood samples and four individuals where positive in oral swab samples. Three of the eight infected were females while the other five individuals had unknown sex. Two of the eight were adults, two were juveniles and the remaining four animals had unknown age. DNA concentration was significantly higher in extracts of blood samples than swab samples, except for FV3-like positive animals in which oral swab extracts (41.3 μg/ml; 95% CI: 11.0 – 56.8) had higher

[2] Content of this chapter has been partially or fully submitted for publication as cited in bibliography as Allender et al., 2012b.

concentration than whole blood extracts (22.5 μg/ml; 95% CI: 14.2 – 100). DNA purity (A260/A280 ratio) was non-significantly more variable for extracts of swab samples than blood samples. Clinical signs significantly associated with infection were diarrhea and bone fractures. Results of this study indicate a low prevalence of disease in box turtles that is characteristic of an acute disease process. Based on this study and other published reports, eastern box turtles are sensitive to frog virus 3 infection, and this virus represents a potential threat.

Key Words: Ranavirus, *Terrapene carolina*, qPCR, chelonian, infectious disease

Introduction

Ranaviral disease has been linked to several mortality events in amphibians and reptiles (Green, 2002; Johnson et al., 2008). These outbreaks have been variable across the landscape, often leading to significant mortalities that may threaten biodiversity (Grey et al., 2011). Reports in chelonians have been fewer than amphibians, but it is unclear whether this is a natural bias or under reporting. When reported, several species of chelonians, notably the eastern box turtle (*Terrapene carolina carolina*), have had severe disease of short duration (Allender et al., 2011).

The eastern box turtle has been downgraded to vulnerable status by the International Union on the Conservation of Nature and Natural Resources (IUCN) (van Dijk, 2011). Significant declines have been observed in numerous areas throughout its range, with an estimated 30% decline over the previous three generations (Swarth and Hagood, 2006; van Dijk, 2011). Specific causes for the decline are not entirely known, but are attributed to human-induced factors including: road and mowing mortality, collection, nest depredation, prescribed burning, disturbance of nest sites by off-road vehicles, and habitat loss (Swarth and Hagood, 2006; Nazdrowicz et al., 2008; Currylow et al., 2011; van Dijk, 2011). While a combination of factors is likely playing a role in the population declines of the box turtle, disease outbreaks due to *Ranavirus* have been emerging across the eastern United States (US) in chelonians and are also listed as a suspected cause of decline by the IUCN (Allender et al., 2011; Johnson et al., 2008; van Dijk, 2011).

Ranavirus is one of five genera from the family Iridoviridae and one of two genera from the family reported to infect reptiles. They are large, icosohedral, DNA viruses, and viruses from this genus have emerged as a significant cause of mortality events in free-ranging herpetofauna throughout the world (Green et al., 2002; Johnson et al., 2008; Mao et al., 1997). Ranaviral

infection was placed on the World Organization for Animal Health list of reportable diseases for amphibians in 2010. Most disease events in chelonians have been associated with FV3-like virus (Allender et al., 2011; Johnson et al, 2008). Epizootics of frog virus 3 have been scattered across several habitats and landscapes in the US; however, disease predictability has not been successful (Allender et al., 2011). To date in the US, ranaviral disease has been diagnosed in seven species of chelonians; this includes ten outbreaks in eastern box turtles across ten different states (Allender et al., 2011b).

The present study estimated the prevalence of frog virus 3 from different populations of eastern box turtles in the US. Our hypotheses were that: 1) Frog virus 3-like disease is associated with an acute mortality events in box turtles; 2) The prevalence of frog virus 3-like DNA in ante-mortem samples collected from box turtles in the US will be low (<5%); 3) Clinical signs of conjunctivitis, ocular discharge, oral plaques, and respiratory distress will be associated with ranaviral infection in box turtles; 4) The prevalence of ranaviral disease will not be associated with age, sex, or weight characteristics; 5) The prevalence in wild animals presented to rehabilitation facilities would be higher than that found in wild caught animals.

Materials and Methods

Sample populations. Turtles sampled for this study were derived from two populations: wild caught, free-ranging turtles and wild turtles presented to rehabilitation facilities. The wild caught, free-ranging turtles were actively sought in their natural habitat, while the turtles presented to rehabilitation facilities represented animals found in an unnatural place, or that were injured or diseased. The wild caught turtles were sampled from 2008 through 2011 from a population near

Oak Ridge, TN, USA (OR). These turtles were actively searched through human visual encounters or canine search (Figure 4.4). Wild turtles presented to rehabilitation facilities were sampled prospectively in 2007, 2010, and 2011 and opportunistically in 2008 and 2009; five different wildlife rehabilitation centers participated: University of Tennessee (UT)(Knoxville, TN, USA), Wildlife Center of Virginia (WCV)(Waynesboro, VA, USA), North Carolina State University (NCSU)(Raleigh, NC, USA), Alabama Wildlife Center (AWC)(Pelham, AL, USA), and the University of Georgia (UGA) (Athens, GA, USA). All procedures were approved by the Institutional Animal Care and Use Committee approval through the University of Tennessee and University of Illinois (UT: 1630-0507, UI: 10057). The sample size used for this study was based on the following assumptions: an FV3 prevalence of 10% in rehabilitation population and 1% in free-ranging population with an alpha of 0.05 and power of 0.8.

Field Methods. Mass of free-living turtles was measured to the nearest gram. Turtles from the rehabilitation facilities were weighed with a variety of devices, and to at least the nearest 1 g. Blood samples (less than 0.8% body weight) were collected from the subcarapacial sinus using a 22 gauge needle and 3 ml syringe (Figure 3). The samples were immediately placed in a lithium heparin coated microtainer (Becton Dickinson, Franklin Lakes, NJ, USA) and mixed by gentle rocking. Oral epithelium and mucus was collected from within the oral cavity using cotton-tipped plastic handled applicators (Fisher Scientific). The swab tips were placed in a 1.7 mL polypropylene microcentrifuge tube (Costar, Corning Inc., Corning, NY, USA) for storage. The blood and swabs were labeled with a unique identification number and remained on-site at -20ºC, batch shipped on ice, and stored at -20ºC until analysis.

Polymerase Chain Reaction. Quantitative PCR was performed as previously described (Allender et al., 2012a). Briefly, DNA was extracted from whole blood and oral swabs using

manufacturer's instructions (QIAmp Blood Mini Kit, Qiagen, Valencia, CA, USA). TaqMan assay was performed using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA), and probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein (MCP) of frog virus 3. All samples were assayed in three technical repeats using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA, USA), analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems), and results averaged.

Clinical Survey. Clinical signs were evaluated by the admitting clinician at each institution and recorded on a standard data sheet provided (Figure 4). The presence or absence of clinical signs compatible with those previously described for *Ranavirus* infection or other non-specific systemic disease were documented. Specific clinical signs included aural abscess, cellulitis, conjunctivitis, cutaneous abscessation, nasal discharge/rhinitis, ocular discharge, oral abscessation, oral discharge/stomatitis, oral plaque, oral ulceration, and respiratory distress. Each sign was scored as absent (0) or present (1). Clinical signs for systemic disease not specifically reported in upper respiratory tract (URT) infections (at present) include fracture of appendage(s) or shell and diarrhea. These signs were also scored as present (1) or absent (0). Most turtles (328) at OR had engaged their plastron hinge, making it impossible to perform a complete physical examination. Furthermore, 63 rehabilitation turtles had no clinical sign recorded.

Statistical Analyses. Descriptive statistics were tabulated for all continuous variables (weight, viral copy number, DNA concentration, DNA purity). Normality was assessed using the Shapiro-Wilk test. Comparisons were made using ANOVA/Kruskal-Wallis and t-test/Mann-Whitney U for within and between group differences for normally distributed and non-normally distributed data, respectively. Overall prevalence proportions (based on qPCR) and their 95%

confidence intervals were computed. In cases where the prevalence estimate was 0, the 95% confidence intervals were calculated with the technique described by Van Belle and Millard (1998). Prevalence proportions were evaluated for equal probabilities using a One-sample Chi-square test. PCR results, natural history characteristics, and clinical signs were performed using McNemar's Chi-square and Fisher's exact. Chi-square or Fisher exact tests were used to evaluate the presence or absence of natural history characteristics or clinical signs by PCR test results. Odds ratios were calculated for each clinical sign based on frog virus 3-like status. Logistic regression models were created for any of the above variables that had a p value <0.1. Hosmer-Lemeshow goodness-of-fit test was used to evaluate model fit. A stepwise approach was used to build the model. Despite multiple comparisons of clinical signs with prevalence, no p value correction was made due to small sample size and risk for Type I error as previously described (McDonald, 2009). Statistical significance was considered when $p<0.05$. All analysis was performed using statistical software (SPSS 20, IBM statistics, Chicago, IL).

Results

There were 606 (wild turtles: 367, 61.1%; rehabilitation turtles: 231, 38.9 %) turtles sampled from 2007 through 2011, with 109 (18.5%) turtles sampled in 2007, 87 (14.6%) in 2008, 7 (1.2%) in 2009, 261 (41.6%) in 2010, and 142 (24.1%) in 2011. There were 38 (6.3%) individuals sampled from UT, 370 (61.1%) from OR, 125 (20.6%) from WCV, 47 (7.8%)from NCSU, 17 (2.8%) from AWC, and 9 (1.5%) from UGA. Five hundred and twenty-five turtles had sampling months recorded; 7 (1.3%) from April, 75 (14.3%) from May, 208 (39.6%) from June, 142 (27%) from July, 24 (4.6%) in August, 46 (8.8%) in September, 20 (3.8%)in October, 2 (0.4%) in November, and 1(0.2%) in December. Of the 522 animals with age and sex

recorded, 199 (38.1%) were male, 248 (47.5%) were female, and 75 (14.4%) were of unknown sex, 458 (87.7%) were adult, 61 (11.7%) were juvenile, and 3 (0.6%) had unknown age recorded.

Institutions, year, sex, and age classes were not sampled with equal probabilities ($p<0.0001$). There was no difference in the age of turtles sampled by institution ($p=0.698$), year ($p=0.460$), or month ($p=0.968$). Juvenile turtles were significantly associated with unknown sex ($p<0.0001$). There was no difference in the sex of turtles sampled by institution ($p=0.093$) or month ($p=0.635$). There were significant differences in the sex of turtles when evaluating year (Figure 4.1) ($p=0.007$). There was a significant difference in the month turtles presented by year (Figure 4.2) ($p<0.0001$) and institution (Figure 4.3) ($p<0.0001$). There were no significant difference in the month turtles presented based on age ($p=0.968$) or sex ($p=0.635$).

Mean weight for females (350 g, 95% CI: 338 – 362) and males (345 g, 95% CI: 335 – 354) was significantly ($p<0.0001$) higher than that of unknown sex turtles (202 g, 95% CI: 174 – 231); there was no significant difference between females and males ($p=0.77$). Adult turtles weighed 353 g (95% CI: 347 – 360), juveniles weighed 131 g (95% CI: 115 – 147), and unknown aged turtles weighed 203 g (Min/Max: 95 – 265). Adults were significantly heavier than juveniles ($p<0.0001$) and unknown age turtles ($p=0.001$). Turtle weight was not significantly different by institution ($p=0.717$).

There were 444 blood samples collected and 434 oral swab samples collected, for a total of 540 turtles with either a blood sample or oral swab or both (400) collected. Mean DNA concentration in blood samples was 161.9 µg/ml (95% CI: 142 – 180) and swab samples 20.7 µg/ml (95% CI: 19 – 22). DNA concentration of blood sample extracts was significantly higher than swab samples ($p<0.0001$), but there was no difference in purity between blood and swab samples ($p=0.195$). Purity of DNA (A260/A280) in blood samples was 1.81 (95% CI: 1.79 –

1.82) and swab samples was 1.91 (95% CI: 1.75 – 2.06). Purity of DNA from swab samples was not significantly ($p=0.262$) different by institution. However, significant differences were seen with DNA concentration of blood samples ($p<0.0001$), DNA concentration of swab samples ($p<0.0001$), and purity of DNA from blood samples. There was a significant difference in DNA concentration of blood samples between NCSU (325 µg/ml) and UT (152 µg/ml) ($p=0.003$), OR (171 µg/ml) ($p<0.0001$), WCV (101 µg/ml) ($p<0.0001$), and AWC (25 µg/ml) ($p<0.0001$). There were significant difference in the DNA concentration from swab samples between OR (26.9 µg.ml) and WCV (15 µg/ml) ($p<0.0001$), OR and NCSU (7 µg/ml) ($p<0.0001$), UT (21 µg/ml) and NCSU ($p=0.003$). There were significant differences in the purity of DNA from blood samples between UT (1.89) and OR (1.81) ($p=0.009$), UT and WCV (1.81)($p=0.014$), UT and NCSU (1.77) ($p<0.0001$), and UT and AWC (1.74) ($p=0.005$).

There were significant differences in DNA concentration from blood samples ($p<0.0001$) and swab samples ($p<0.0001$) by year. DNA concentration in blood samples was higher in 2007 than 2008 ($p<0.001$), 2010 ($p<0.001$), and 2011 ($p<0.001$). DNA concentration in swabs samples was lower in 2007 compared to 2008 ($p=0.014$), 2010 ($p=0.001$), and 2011 ($p<0.001$); as well as 2008 compared to 2011 ($p=0.03$). There no differences in weight ($p=0.188$), DNA purity from blood samples ($p=0.328$), or DNA purity from swab samples ($p=0.579$) by year (Table 4). There were significant differences in weight ($p<0.0001$) and DNA concentration from blood between sexes (female: 171.5 µg/ml; male: 152.6 µg/ml; unknown: 234.14 µg/ml). There were no differences between sexes for DNA concentration from swab samples ($p=0.352$), DNA purity from either blood ($p=0.090$) or swabs ($p=0.853$).

Turtles presented to rehabilitation centers had a higher prevalence of FV3-like infections than free-living box turtles ($p=0.01$). There were four positive swab samples and four positive

blood samples, no turtle was positive on both sample types despite both sample types being tested in each animal. There were no significant differences in FV3-like status by year (p=0.493; observed power ranged 0.02 through 0.42), sex (p=0.156; observed power ranged from 0.05 through 0.76), or age (p=0.073; observed power = 0.9). UT, WCV, NCSU, and AWC had higher prevalence than UGA or OR (p<0.0001). When month was recorded, FV3-like prevalence was detected in April (n=1), June (n=2), and July (n=1) which were not significantly different. However, FV3-like prevalence was different between those months and the months that no FV3-like was detected (p=0.04). There was no association of FV3-like status with year (p=0.515) (Table 2), age (p=0.081) (Table 3), or sex (p=0.157) (Table 3). Logistic regression modeling for overall FV3-like status that included independent variables of institution, month, and age was not significant (p=0.260). Logistic regression modeling for FV3-like prevalence in blood that included independent variables of year, month, and institution was not significant (p=0.338). Logistic regression modeling for FV3-like prevalence in swab that included independent variables of age, institution, and sex was not significant (p=0.796). Weight was significantly lower in ranavirus-positive animals (198g) than ranavirus-negative animals (325 g) (p=0.012).

Two hundred and eight animals had full physical examinations performed. There were significant associations with FV3-like presence and fracture (n=3) (p=0.045) and diarrhea (n=1) (p=0.017) when no Bonferroni correction was made. However, neither clinical sign was significant when the correction was made. The other clinical signs that were evaluated were not found to be significantly associated with FV3 prevalence: aural abscess (p=0.895), cellulitis (p=0.967), conjunctivitis (p=0.934), cutaneous abscessation (p=0.983), lethargy (p=0.902), nasal discharge (p=0.910), ocular discharge (p=0.910), oral discharge (p=0.934), oral plaque

(p=0.967), or respiratory distress (p=0.910). FV3-like positive turtles were 153 times more likely (95% CI: 4.7 – 4942) to have diarrhea than in FV3-like negative turtles.

There were no differences in weight (p=0.134), DNA blood concentration (p=0.243), or DNA purity in blood (p=0.242) based on FV3 prevalence in blood (Table 4). There was a significant difference in weight (p=0.21) between ranavirus positive (positive: 325 g) and negative turtles (198 g). There were no differences in DNA concentration in swabs (p=0.067) or DNA purity (p=0.928) based on FV3-like status (Table 4).

Median FV3-like virus copy number from blood samples in positive animals was 1927 (Min/Max: 3854 – 28060) not significantly different from median FV3-like virus copy number from swab samples in positive animals was 2505 (Min/Max: 5011 – 29893) (Table 4).

There were 13 (6.3%; 95% CI: 3.7-10.4%) animals with an aural abscess, 4 (1.9%; 95% CI: 0.8-4.8%) with cellulitis, 8 (3.9%; 95% CI: 1.9-7.4%) with conjunctivitis, 115 (55.8%; 95% CI: 48.5-61.9%) with fracture, 11 (5.3%; 95% CI: 2.9-9.2%) with lethargy, 11 (5.3%; 95% CI: 2.9-9.2%) with nasal discharge, 11 (5.3%; 95% CI: 2.9-9.2%) with ocular discharge, 8 (3.9%; 95% CI: 1.9-7.4%) with oral discharge, 4 (1.9%; 95% CI: 1.9-7.4%) with oral plaques, and 11 (5.3%; 95% CI: 2.9-9.2%) with respiratory distress. Other clinical signs seen in single animals included tongue discoloration, rear limb ataxia, edema of the neck and forelimbs, and skin lacerations. There were three animals that were positive for FV3-like DNA in blood that had clinical signs recorded. None of these three positive turtles had aural abscess, cellulitis, conjunctivitis, lethargy, nasal discharge, ocular discharge, oral plaque, or respiratory distress. However, all three animals had a fracture and one of the animals had diarrhea. None of turtles with FV3-like virus positive swab samples had clinical signs recorded.

Discussion

As populations of box turtles decline, the opportunity for disease-related events to lead to catastrophic events increase. A disease that has a short duration of effect with high mortality may lead to such a catastrophic event. Thus far, over 70 reports of *Ranavirus* infections in free-ranging amphibians have indicated that mortality rates may be as high as 90% locally, with potential to cause local extirpations (Jancovich et al., 2003; Miller et al., 2011). Large events such as these have not been documented in chelonians, and it is unknown whether they are occurring or going unnoticed. However, there have been at least sixteen reports of ranaviral disease in chelonians, with 10 of them involving the eastern box turtle (Allender et al., 2011b; DeVoe et al., 2004; Allender et al., 2011a; Johnson et al., 2008; Ruder et al., 2008; Johnson et al., 2010). Most of these represent single case reports of less than ten individuals involved in an outbreak. This is not surprising as the solitary behavior and habitat of the turtle may prohibit multiple animals from being detected. This is in contrast to amphibians that breed in ephemeral ponds and are commonly seen in high densities. It would be of great concern if the same level of mortality was occurring in box turtles, but was going unnoticed because of these natural history characteristics. The solitary behavior of turtles may prevent or slow the spread of disease if infrequent intra-species contact occurs, but high level of interactions may still occur during certain times of the year (e.g. mating).

The prevalence across several states was variable, but was confirmed in Alabama, Tennessee, and Virginia. The prevalence was highest in Alabama, which was significantly higher than all other institutions except UT, but is likely due to the small sample size tested from that state. Frog virus 3-like infection in eastern box turtles have previously been seen in Tennessee and Virginia (Allender et al., 2011a), but this is the first report from Alabama. The low

prevalence in this study is similar to that reported for gopher tortoises and box turtles using an ELISA (Johnson et al., 2010). This low prevalence in combination with a high mortality reported in chelonians (Johnson et al., 2006; Allender et al., 2012c) reinforces that this is a disease with an acute duration and continued monitoring is needed to document range expansion of frog virus 3 in eastern box turtles.

Larval and metamorphic amphibians of most species have higher documented mortality to FV3 than adult amphibians (Miller et al., 2011), but no such information is available for chelonians. In this study, we found that juvenile turtles were more likely to be infected with frog virus 3 than adults. However, there were only 57 juveniles tested, while 400 adults were tested. Juvenile turtles are more difficult to find, and may represent a highly susceptible age class that should be monitored more closely. Juvenile box turtle survivorship is reported to be low (Dodd, 2001), and threats that further reduce it may threaten long-term sustainability.

There was a statistically significant difference in prevalence of frog virus 3-like virus based on year, with 2008 higher than 2007, 2009, and 2011. However, this observation is not expected to represent a biologically significant finding, as the prevalence remained low in all years. The year with the lowest prevalence also had the lowest number of animals tested. And even though there are several amphibian populations that have endured annual outbreaks (Fox et al., 2006; Carey et al., 2009), this pattern has not been seen in chelonians. The absence in certain years likely represents sampling bias rather than the virus being absent from the environment. This study found two cases in June, and one each in April and July, and four cases with no month recorded. Differences in infectivity of ranaviruses due to temperature have been shown for amphibians (Rojas et al., 2005) and turtles (Allender et al., 2012c), which likely will lead to differences in detection based on season and environmental temperature. Future studies need to

identify the time of year and locations of known cases and begin ongoing surveillance of those locations to further characterize the epidemiology in relation to environmental temperature. The ability to target a season or temperature range that turtles are likely to be infected may aid in management efforts of threatened or endangered populations and increase the probability of finding infected turtles.

There were three females and no known males diagnosed with frog virus 3-like in the current study. Five positive animals had no sex recorded and were not included in the analysis of sex as a factor in FV3-like status. There has been no sex predilection described in other reports on amphibians or reptiles and the results of this study are likely only a statistical anomaly.

This was the first study to utilize quantitative PCR to survey populations of chelonians. This method is considered more sensitive than conventional PCR, allowing for the detection of potential subclinical disease states. However, there has been no evidence that box turtles exist in a subclinical state. The severity of disease and high mortality in this species indicates that if the animal is exposed, mortality occurs. The eight animals that tested positive in this study were lost to follow-up and confirmation that these animals died from ranavirus would need to be confirmed based on necropsy evaluation. Previous reports have demonstrated that the virus is detectable in many different tissues post-mortem (Johnson et al., 2007); however, clinical antemortem samples have typically involved whole blood or swabs of the oral mucosa. This study utilized both blood and oral swabs and viral copy number was not significantly higher in blood than oral swabs. Previous studies have shown that samples that are positive in blood are also positive in swabs (Allender et al., 2011), but qPCR development in turtles indicated that detection in swabs may occur prior to blood (Allender et al., 2012). In this study, there were eight positive animals detected with four positive in blood and four positive in oral swabs. There

are several factors that may have lead to one sample being positive and the other sample negative, such as inefficient DNA extraction, poor sample collection, inappropriate sample handling, or absence of viral shedding in the sample. In experimental transmission in turtles, infected animals demonstrate viremia from initial detection through death and therefore absence of shedding is not likely the cause of the negative status in blood samples. Additionally, the detection in swab samples only may represent the mode of transmission, which is unknown in chelonians. If transmission occurs through an oral route, then DNA might be detected in the oral swab sample prior to blood. Regardless, future investigations should continue to sample multiple tissue types to increase detection ability in a population.

One molecular factor that may lead to false negatives includes the DNA concentration and DNA purity. This study utilized a spectrophotometer that allowed for the quantification of DNA and assessment of its purity by using 1 µl of sample. This technology allows the user to assess whether the extraction was ideal for the developed PCR. If not ideal, then purification of the extract can be performed. However, it doesn't allow differentiation of viral DNA or host DNA. There was no statistically significant difference in DNA quantity or purity between blood and swab samples that were positive for FV3 in the current study, however DNA concentration was higher in positive swabs rather than positive blood. Similarly, DNA concentration in positive blood was lower and concentration of positive swabs was higher than the negative samples. Positive blood may have less DNA because of disease-related anemia or increased total solids in samples that dilute total DNA concentration. However, decreases in total solids have been seen with experimental transmission of FV3-like infection in red-eared sliders (Allender et al., 2012d). Positive swab samples may have had more DNA because there was increased cell

sloughing that was disease-related or other pathogens such as bacteria, other viruses, and parasites.

Manufacturer's instructions indicate that the ideal range for DNA of A260/A280 is 1.7 to 1.9, and that this represents a pure sample. Swab extracts were much more variable than blood samples despite the fact that the median value was nearly the same as the median value of the blood extracts. This is likely due to less DNA in the sample or inhibitors in the cotton on the applicators. Conversely, blood samples were more frequently purified because of impure original extraction. This impurity is likely due to the overwhelming amount of host DNA and cell lysis that is required in samples with nucleated blood cells. All of these blood samples were isolated based on 200 µl of whole blood sample, however, recent manufacturer's recommendations indicate that 10 µl of whole blood is the current volume that should be used for samples with nucleated red blood cells. This will undoubtedly decrease not only the potential host DNA that is available for extractions (as is anticipated by the manufacturer's changes), but it will consequently decrease the potential viral DNA. Alternatively, extractions can use higher volumes of blood, but require more reagents and time for extraction. Future studies should determine if smaller sample volumes can be optimized that maximize detection of FV3.

Several clinical signs have been associated with FV30like infection in turtles, including ocular discharge, oral discharge, respiratory distress, and oral plaques (DeVoe et al., 2004; Johnson et al., 2006). Unfortunately, none of those clinical signs were associated with positive status in this study. The absence of significant associations with clinical signs is likely due to the low prevalence of ranavirus and the relatively high number of clinical signs in non-infected animals. Despite this low prevalence, both fracture and diarrhea were significantly associated with FV3-like infection in this study. This was a biased population in that several rehabilitation

clinics were included, but this allowed animals with fractures to be identified with FV3-like infection. This is valuable to clinicians at these or other rehabilitation clinics to not overlook infectious diseases in the face of the fracture. Diarrhea has high odds of occurring with FV3-like infection. This likely represents a manifestation of systemic disease rather than a specific disease process. The failure of this study to identify other clinical signs indicates that other signs are non-specific and several other FV3-like negative turtles were exhibiting oral plaques, ocular discharge, and nasal discharge. All future studies should continue to record clinical signs, it may be possible to better determine associations with clinical signs in populations that have higher prevalence.

In summary, this was the largest cross-sectional study to estimate the prevalence FV3-like virus in wild and rehabilitating eastern box turtles. It demonstrated that the prevalence of FV3-like virus is low in both populations, and that temporal and geographical influences were minor. Findings that FV3-like virus was in non-significantly higher proportion in females and juvenile animals warrant further work, especially if there is a potential for vertical transmission. Clinical signs of fracture and diarrhea were significantly associated with infection. Since fracture is a common finding in turtles presented to rehabilitation facilities (Brown and Sleeman, 2002; Schrader et al., 2010), this finding may have been biased by the types of presentations. It is also possible that animals infected with a virus that causes acute disease with a high mortality could affect the behavior of the turtle and cause it to be more likely to become injured (e.g., hit by car). Infection was detected both in blood and oral swabs, and future studies should continue to use these samples for detection. These findings elucidate that FV3-like infection has several signs of an acute systemic disease: low prevalence, few clinical signs, and no biologically significant sex-specific prevalence. The higher prevalence in juveniles is supported by other studies that show

higher mortality in juvenile salamanders (Miller et al., 2011), but more work is needed to determine if this is true in experimental settings as well.

Eastern box turtles presented to rehabilitation centers are more likely to be detected with FV3-like infections than free-living eastern box turtles. The presence of FV3-like in free-ranging animals may indicate a threat to population sustainability and future research is needed to determine the impacts to endangered populations.

Table 4.1. Prevalence of frog virus 3-like DNA determined by quantitative PCR in eastern box turtles sampled from the southeastern US.

Location		FV3-like positive	FV3-like negative	Prevalence	95% CI
Free-living*		1	308	0.3%	0 – 1.8 %
Rehabilitation*		7	217	3.13%	1.5 – 6.3%
	UT	3	35	7.9%	2.7 – 20.1 %
	WCV	2	120	1.6%	0.4 – 5.9 %
	NCSU	0	46	0.00%	0 – 7.7 %
	AWC	2	14	14.3%	4.0 – 39.9 %
	UGA	0	9	0.00%	0 – 29.9 %
Total		8	532	1.5%	0.8 – 2.9 %

* Significantly higher prevalence in rehabilitation turtles (p=0.01) than free-living turtles.

Table 4.2. Prevalence of frog virus 3-like virus in blood and oral swab samples determined by quantitative PCR in eastern box turtles from 2007-2011.

Year	FV3-like positive	FV3-like negative	Prevalence	95% CI
2007	1	107	0.9 %	0.2 – 5.1 %
2008	3	80	3.6 %	1.2 – 10.1 %
2009	0	7	0.0%	0 – 35.4 %
2010	3	207	1.4%	0.5 – 4.1 %
2011	1	131	0.8%	0.1 – 4.2 %

Table 4.3. Prevalence of frog virus 3-like virus in blood and oral swab samples determined by quantitative PCR in eastern box turtles based on age class and sex from 2007-2011.

Variable	FV3-like positive	FV3-like negative	Prevalence	95% CI
Female	3	168	1.8 %	0.6 – 5.0 %
Male	0	218	0.00%	0 – 1.7 %
Adult	2	398	0.5 %	0.1 – 1.8 %
Juvenile	2	55	3.5 %	0.9 – 11.9 %

Table 4.4. Descriptive statistics for DNA concentration and DNA purity (A260/A280 ratio) for extracts of blood and oral swab samples from eastern box turtles from 2007 through 2011.

	Blood			Swab		
	Median	10-90% percentiles	Min/Max	Median	10-90% percentiles	Min/Max
FV3-like virus copy	13297	3854 - 24287	3854 - 28060	6871	5011 - 24337	5011 - 29893
DNA concentration	78.1[#]	19.5 - 401.2	2.7 - 1472.6	14.9[#]	3.7 - 43.8	0.32 - 132.9
DNA purity	1.84	1.65 - 1.89	0.78 - 2.77	1.83	1.4 - 2.15	0 - 25.7

[#] Statistically significant higher in blood samples, $p<0.0001$

Table 4.5. Descriptive statistics for DNA concentration and DNA purity (A260/A280 ratio) for extracts of blood and oral swab samples from eastern box turtles from 2007 through 2011 with or without molecular evidence for frog virus 3.

		FV3-like Positive			FV3-like Negative		
		Median	10-90% percentiles	Min/Max	Median	10-90% percentiles	Min/Max
Blood	DNA concentration	22.5	14.1 - 100	14.1 - 124.7	97.2	19.4 - 495.4	2.7 - 1472.6
	DNA purity	1.75	1.57 - 1.86	1.57 - 1.88	1.84	1.67 - 1.89	0.78 - 2.77
Swab	DNA concentration	41.3	11.0 - 56.8	11 - 57.2	14.5	3.5 - 43.85	0 -132.6
	DNA purity	1.94	1.85 - 2.14	1.85 - 2.2	1.83	1.39 - 2.14	0 -25.7

Figure 4.1. Distribution of eastern box turtles by sex and year evaluated for frog virus 3-like virus.

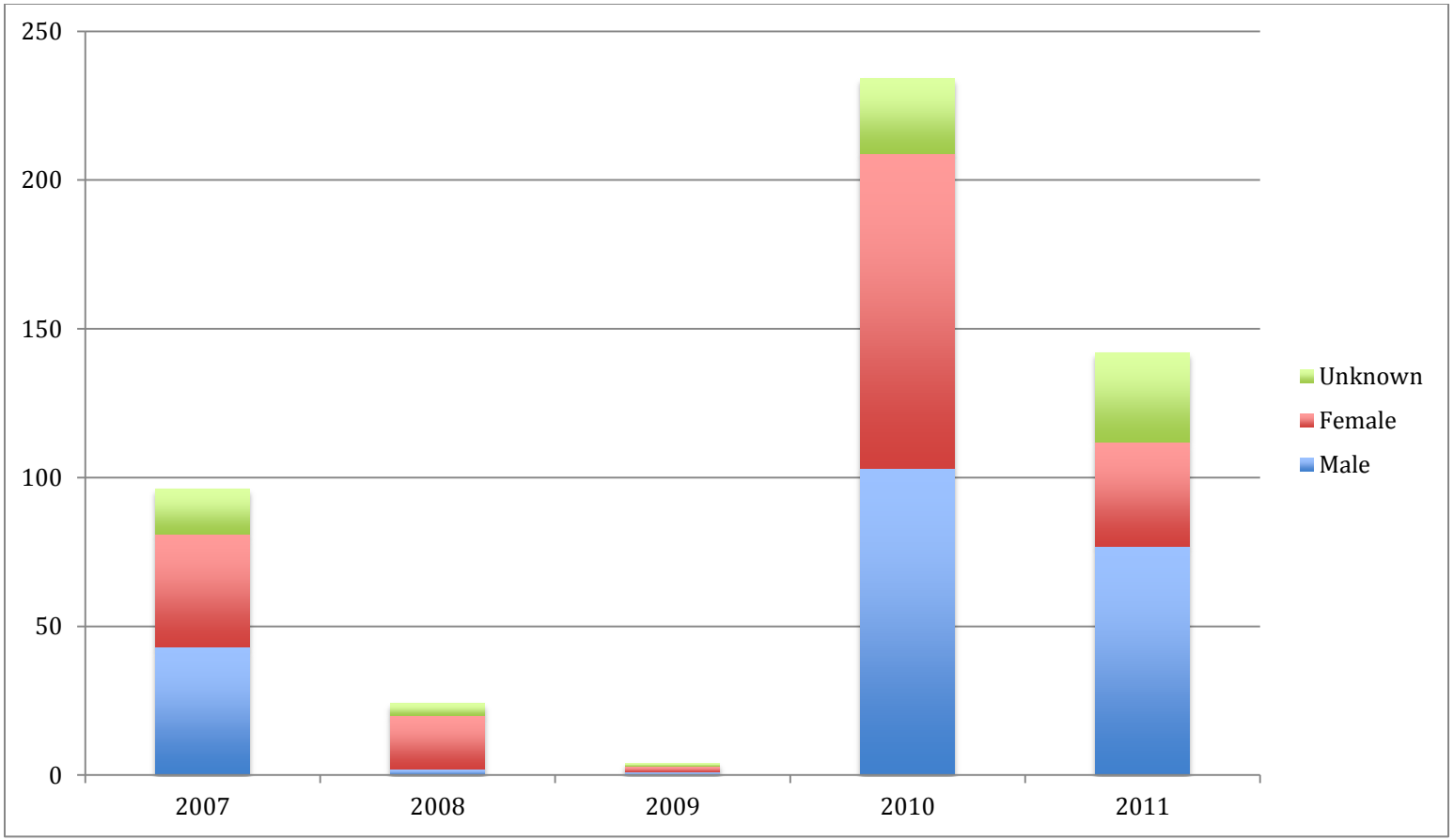


Figure 4.2. Proportion of eastern box turtles that presented each month by year.

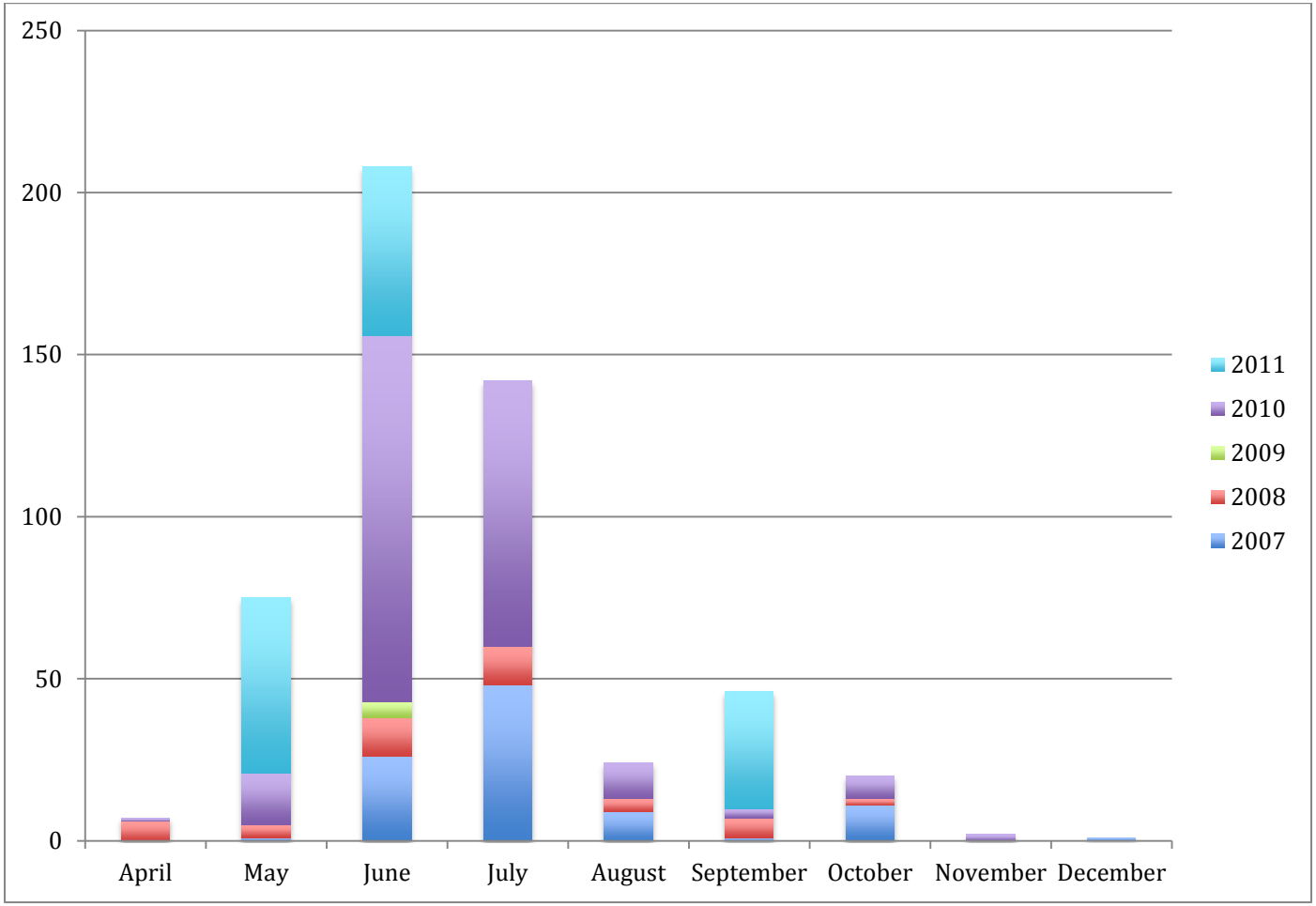


Figure 4.3. Proportion of eastern box turtles that presented each month by institution.

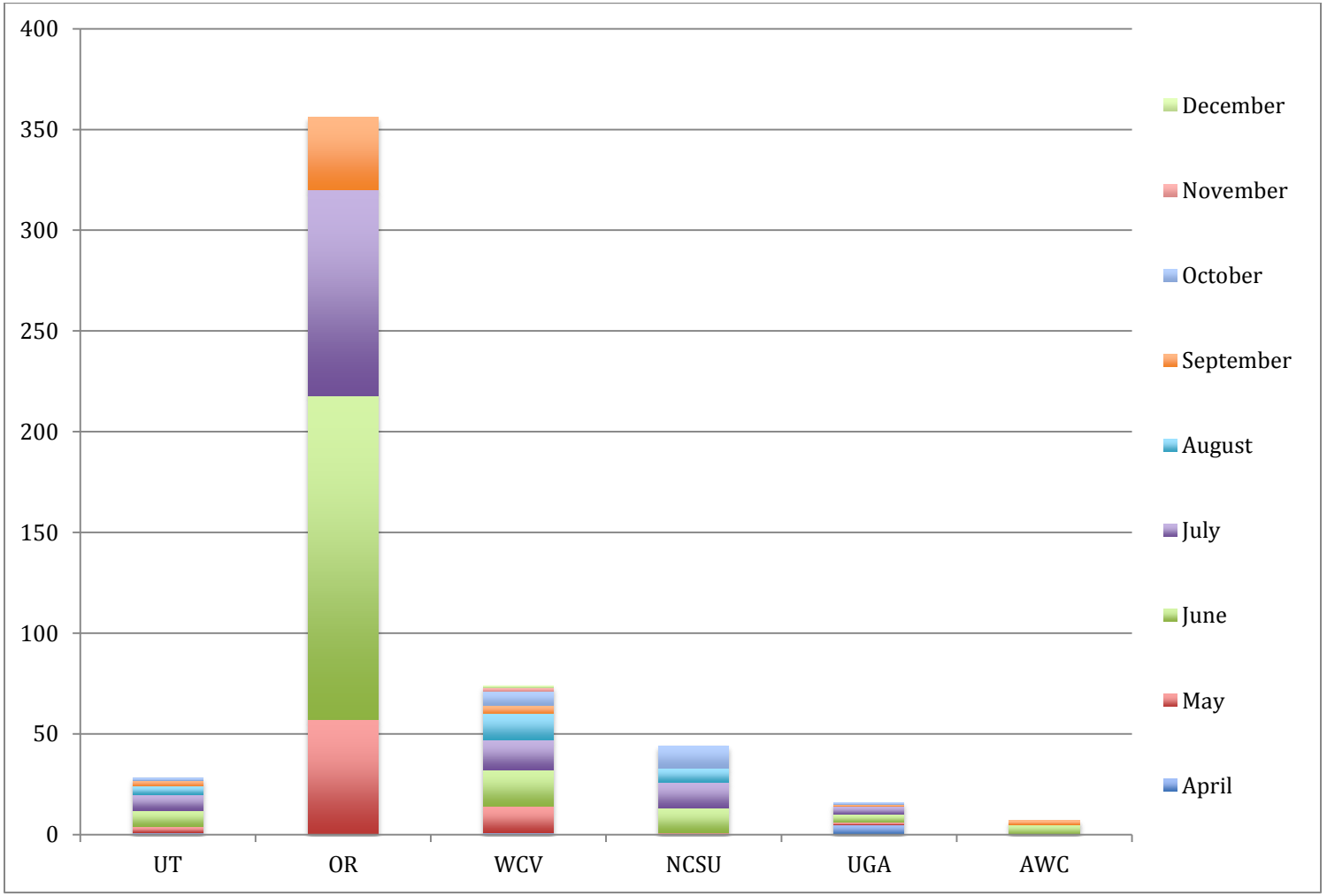
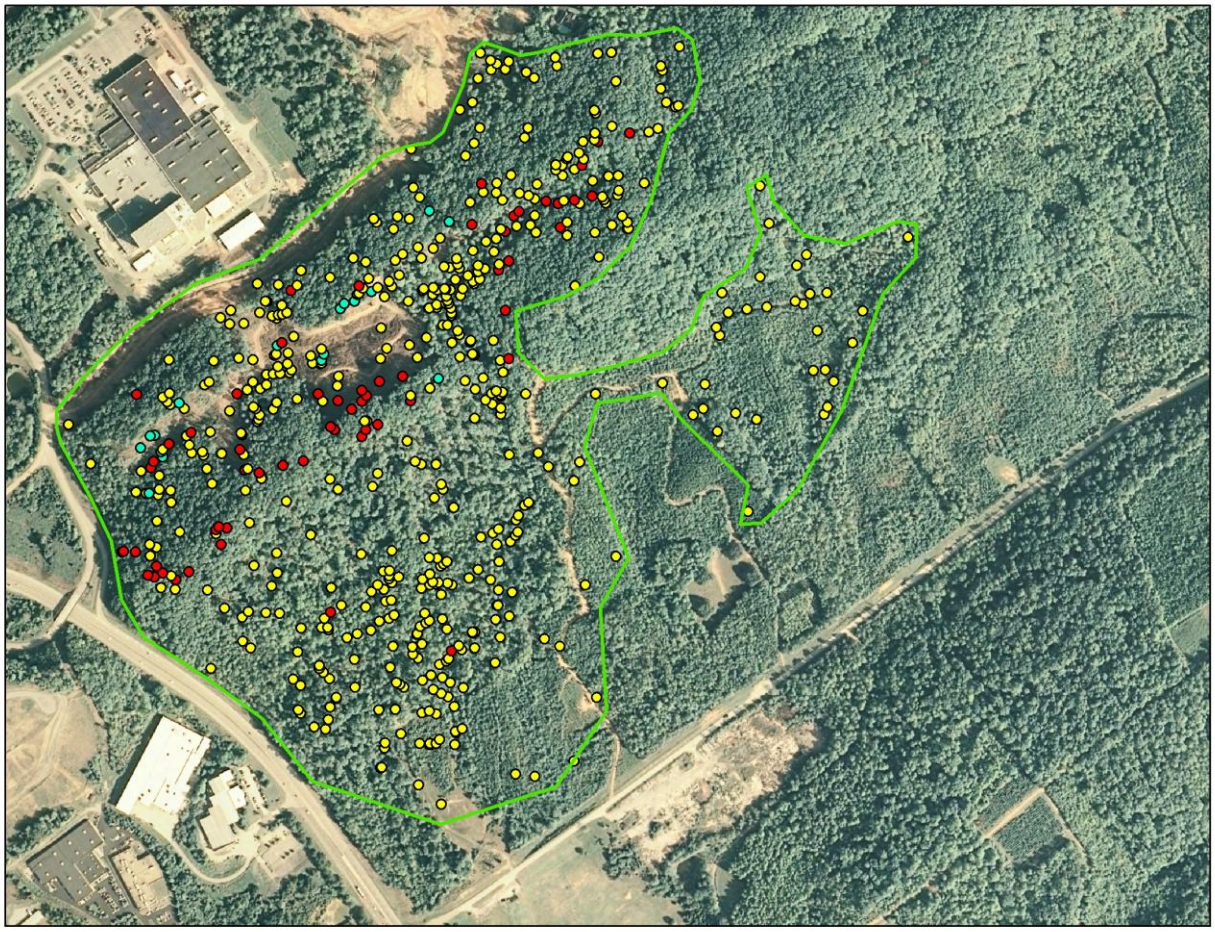


Figure 4.4. GPS locations of individual eastern box turtles observed using canine searches (yellow dot), incidental human captures using 50m x 50m transect searching (red dot), and incidental human encounters (turquoise dot) from 2006 through 2008 at the Oak Ridge, TN site.



CHAPTER 5

EVALUATING THE EFFECTS OF EXPERIMENTAL FROG VIRUS 3-like virus ON RED-EARED SLIDERS (*TRACHEMYS SCRIPTA ELEGANS*) HELD AT DIFFERENT ENVIRONMENTAL TEMPERATURES [3]

Abstract: Ranaviral disease has been reported in several species of reptiles, but the disease progression and mortality in relation to environmental temperature has yet to be determined. In this study, adult female red-eared slider turtles (*Trachemys scripta elegans*) were evaluated after experimental inoculation with a ranavirus isolate at 22°C and 28°C. Mortality at 22°C was 100% (4/4), while mortality at 28°C was 50% (2/4). Median survival time for turtles exposed to 22°C was 24 days, while it was 30 days in the 28°C trial. Consistent histologic lesions were observed only in the 22°C inoculated turtles and included fibrinoid necrosis of vessels in the spleen, vascular and sinusoidal thrombi in the liver, necrotizing myositis, and a mild heterophilic interstitial pneumonia. Quantitative PCR was able to detect viral copies in whole blood, oral and cloacal swabs, tongue, skeletal muscle, lung, heart, liver, spleen, ovary, and kidney in all affected individuals of both trials. Viral copy number in antemortem clinical samples was non-significantly highest in whole blood compared to oral swabs and cloacal swabs, while kidney had the non-significantly highest viral copy number in postmortem samples. Means of each sample type had non-significantly higher viral copy number in turtles exposed to 22°C compared to 28°C. This study determined that environmental temperature affects the survival and disease

[3] Content of this chapter has been partially or fully submitted for publication as cited in bibliography as Allender et al., 2012c.

progression in ranavirus-infected red-eared sliders, which will aid in managing animals in a clinical or free-ranging setting.

Key Words: chelonian, reptile, iridovirus, transmission, pathogenesis

Introduction

Wildlife diseases have been on the rise across the world (Daszak et al., 1999). It is important to develop an understanding of these disease events as they can have a dramatic impact on local populations (Daszak, 1999). Over 80 epizootics, on five continents, specifically involving ranaviruses in reptiles and amphibians have been reported in the literature since 1991, (Miller et al., 2011). A majority of the epizootics have involved amphibians; however, there has been an increase in the number of reports in chelonians, and specifically box turtles, in the US (Johnson et al., 2008; Allender et al., 2006, DeVoe et al., 2004).

Ranaviruses, members of the family *Iridoviridae*, are large, icosohedral, DNA viruses that can be found in an enveloped or non-enveloped form (Chinchar, 2009). Frog virus 3 (FV3) is the type species in the genera, and FV3-like virus has accounted for all of the known cases of disease in chelonians. Disease events have been scattered between captive and free-ranging animals with no apparent pattern, and much of the epidemiology of this virus in chelonians is unknown.

A previous report describing an experimental challenge in red-eared slider turtles (*Trachemys scripta elegans*) fulfilled Koch's postulates, establishing FV3-like as the causative agent of disease in a chelonian (Johnson et al., 2007). The study, however, did not evaluate the effect of temperature on the development of disease. Temperature has been shown to have a profound impact on survival and pathology in iridoviral infections in fish and amphibians (Rojas et al., 2005; Jun et al., 2009), and merits study in reptiles.

The purpose of this study was to evaluate the role of temperature in the pathogenesis of FV3-like infection in red-eared slider turtles. The specific biological hypotheses tested in this study included: 1) Turtle survival will be higher in FV3-like infected animals maintained at a

higher environmental temperature; 2) Less severe pathological changes will be noted in FV3-like infected turtles maintained at higher environmental temperatures; 3) Viral copies from oral and cloacal swabs, whole blood, and necropsy tissues collected from FV3-like virus infected turtles will be lower in turtles maintained at a higher environmental temperature ; and 4) There will be no significant difference in viral copies between any ante-mortem sampling method (oral swab, cloacal swab, whole blood).

Materials and Methods

Animals and husbandry. All activities were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol: 11050). Animals in this study were concurrently sampled as part of a separate study (Allender et al., 2012c). Sixteen adult female RES were acquired from a commercial turtle farm. Using computer-generated randomization, four animals were assigned to receive treatment and four animals were assigned to remain as uninfected controls in each of the two environmental temperature trials. The first trial was carried out in a 15'9" x 12" environmental chamber held at 22°C. This trial was carried out with both the inoculated and control animals in the same room separated by a double plastic barrier and 2-foot hallway created between the plastic barriers. The second trial was carried out with the inoculated and control animals housed in separate, but identical, environmental chambers at 28°C. Each temperature trial included a seven day acclimatization period and was terminated at 31 days post-inoculation. The first trial began in July 2011 and the second began in November 2011. Animals were housed singly in 45 or 50 gallon plastic enclosures with 20 gallons of water and access to a dry dock (cinder block). Animals were fed a commercial aquatic turtle diet (Fluker Farms, Port Allen, LA) every other day.

A ranavirus isolated from an infected eastern box turtle was grown in *Terrapene* heart cells (TH-1). Flasks were scraped when cells exhibited 100% cytopathic effects. Cells and media were then transferred to 15ml tubes, centrifuged at 4,500x*g* for 30 minutes, and the supernatant discarded. The cell pellets were resuspended in residual media, then frozen and thawed three times, thoroughly vortexed before and after each freeze cycle, and centrifuged again at 4,500x*g* for 30 minutes. DNA was extracted, conventional PCR utilized to amplify a 531 bp segment of the major capsid protein, and sequenced to be 100% homologous to FV3. Quantitative PCR was then performed as previously described to quantify viral DNA. Viral titers were determined using serial dilutions of virus in cell culture.

At the start of the study each animal was examined and weighed; blood (1.5 mls), oral and cloacal swabs (plastic handled cotton-tipped applicator, Fisher Scientific) were collected 7 days (-7 d) and 4 days (-4 d) before the virus challenge. Blood samples were collected from the subcarapacial sinus. Quantitative PCR was performed on the whole blood and both swabs from both days to confirm negative status. On day 0, each treatment animal was given 5×10^5 $TCID_{50}$ FV3-like virus intramuscularly in the right forelimb. Each control animal was administered an equal volume of uninfected TH-1 cell lysate on the same day. Each animal was weighed and clinical samples (blood, oral and cloacal swabs) were collected from each animal on days 3, 7, 10, 14, 17, 21, 24, 28, and 31 post-inoculation. Whole blood was immediately placed in two lithium heparin microtainers, while the swabs were each placed in separate 2.0 ml polypropylene eppendorf tubes. All samples were stored at -20°C until analysis.

Clinical signs. Clinical signs were evaluated daily and recorded. The presence or absence of clinical signs compatible with those previously described for *Ranavirus* infection or other non-specific systemic disease were documented. Specific clinical signs evaluated daily included:

cutaneous abscessation, nasal discharge, ocular discharge, oral plaque, and lethargy. Other less common clinical signs were recorded as they occurred for each animal. Each sign was scored as absent (=0) or present (1).

Quantitative PCR. Quantitative PCR was performed as previously described (Allender et al., 2012a). Briefly, DNA was extracted from whole blood, oral, and cloacal swabs using manufacturer's instructions (QIAmp Blood Mini Kit, Qiagen, Valencia, CA). Tissues collected at necropsy were weighed and DNA extracted following manufacturer's instructions (DNeasy kit, Qiagen). TaqMan assay was performed using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA), and probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein (MCP) of FV3. All samples were assayed in three technical repeats using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA), analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, CA), and results averaged.

Pathology. Turtles were euthanized when clinical signs of infection became severe (dyspnea, open-mouth breathing, weight loss greater than 20%/week or at 30d post-inoculation). Ketamine (100mg/kg) was administered intramuscularly in the left forelimb, followed by an overdose of pentobarbital (Fatal-Plus, 2ml total dose) in the subcarapacial sinus after the animal had lost consciousness. Each animal was necropsied separately under a biological safety hood within 3 hours, except for one turtle that was necropsied within 48 hours for reasons beyond control. There was no histopathologic evidence of autolysis in that turtle. Sterile procedures were used to collect samples of tongue, right forelimb skeletal muscle, liver, heart, lung, spleen, kidney, and ovary for qPCR. Duplicate samples of tissues were collected and placed in formalin. Samples were processed routinely, sectioned at 3 µm, and stained with hematoxylin and eosin. Presence

and severity of histopathological abnormalities associated with ranaviral disease, such as intracytoplasmic inclusions and necrosis, were evaluated. Initially, all slides were subjectively evaluated to determine if a lesion scoring method was feasible. The pathologist was blinded to the treatment and control status of the turtles in each temperature group.

Statistical analysis. Animals were classified as either inoculated (administered FV3-like virus) or control (administered uninfected cell lysate), then further analyzed as either infected (developed disease and qPCR presence) or uninfected (did not develop disease or qPCR presence) for each temperature trial. Sample size was determined using the a priori information of alpha=0.05 and Power=0.8 to detect a significant difference in disease prevalence if 75% of the inoculated animals are infected and less than 25% of the control animals are infected. Descriptive statistics were produced for each continuous variable (weight, viral copy number) at each time point, including the mean/median, 95% confidence interval/10-90% percentiles, and minimum/maximum. Normality of data was determined using the Shapiro-Wilk test. Associations between disease status and categorical variables (clinical signs, sample type) were assessed using Fisher's Exact/Chi-squared test. In the experimental study, due to missing values from individuals that died due to the study design, four time points were included in the final analysis: the "pre-" sample (two pre-inoculation samples were averaged), the immediate "post-"inoculation sample, and the "terminal" sample (the last sample taken prior to death). The timing of the pre- and the post-samples were the same days for every RES; however, the final sample was collected at the last time point prior to animal death (day 7- 31 post-inoculation). Repeated measures (Friedman's for non-normally distributed data or repeated measures ANOVA for normally distributed continuous variables described above) analysis was performed over time, independent of treatment and temperature exposure in RES. Then, separately, the same analysis

was performed for turtles in each temperature and RES treatment trial, respectively. The sensitivity and specificity to detect ranaviral infection in clinical samples was determined based on the gold standard of the necropsy results. Repeated measures analysis of categorical variables (clinical signs) were evaluated using a Cochran's Q test, and, if significance was found, specific time point differences were further evaluated using the McNemar's test. Differences between temperatures (22°C and 28°C) for continuous variables (listed above) at the same time point were evaluated using an independent samples t-test. Life tables were constructed for days to survival in inoculated turtles and those individuals that survived to the completion of the study were censored. Kaplan-Meier estimates were used to determine any difference in survival based on environmental temperature. Statistical significance was considered for all p values <0.05. All analysis was performed using commercial software (SPSS 20, IBM statistics, Chicago, IL).

Results

Animals and viral isolate. All turtles were grossly examined and deemed apparently free of disease at the start of the study. Duplicate oral and cloacal swabs and whole blood samples collected 7 days and 4 days prior to inoculation were negative for FV3-like DNA.

Survival. Median survival time for red-eared slider turtles experimentally inoculated with FV3-like disease was 24 days (Min/max: 14 to 30 days) for animal maintained at 22°C and 30 days (Min/max: 17 to 30 days) for animals maintained at 28°C. All inoculated turtles in the 22°C were euthanized due to the severity of clinical signs observed, while only two (50%) animals were euthanized in the 28°C trial. The remaining two inoculated animals in the 28°C trial survived with no apparent clinical signs and were euthanized at the termination of the study (30 days). One control animal (animal 6) died in the 28°C study due to bacterial septicemia and was

negative for ranavirus. Survival estimates are shown in Figure 5.1. There was no significant difference in survival between environmental temperatures (p=0.346). When combining both temperature trials, there was a significant association between inoculation and infection (p=0.003). For turtles in the 22°C study, there was a significant difference in infection between inoculated and control animals (p=0.014). There was no significant difference between inoculation and infection in turtles from the 28°C study (p=0.214)

Natural history characteristics. Weights of red-eared sliders were compared between temperatures and over time. There were no significant differences in weights between temperatures at the same time points (pre-inoculation p=0.124; initial post-inoculation p=0.133; terminal p=0.147) between temperature trials. There also was no significant difference in weight at any one point between inoculated and control turtles during the 22°C study (pre: p= 0.497; post: p=0.432; terminal: p=0.313) or 28°C study (pre: p= 0.660; post: p=0.941; terminal: p=0.900), although there was a significant trend in the infected animals at 22°C to increase over time and no significant trend in the control turtles (Table 5.1).

Molecular characteristics. There were no significant differences in DNA concentration between temperature for whole blood (pre: p=0.816; post: p=0.498; terminal: p=0.341), oral swabs (post: p=0.708; terminal: p=0.708), or cloacal swabs (pre: 0.540; post: p=0.669; terminal p=0.333) at either temperature over the entire series of sampling. There was significantly higher DNA concentration in oral swabs of inoculated turtles than control turtles for the pre-inoculation sample (p=0.009). There were no significant differences in DNA concentration over time within whole blood (p=0.057, Power=0.562), oral swabs (p=0.285, Power=0.180), or cloacal swabs (p=0.332, Power=0.155). Results of DNA concentration and purity (A260/A280 ratio) are summarized in Table 5.2.

Quantitative PCR. All turtles inoculated in the 22°C trial were detected with FV3-like DNA in whole blood, oral swab samples, cloacal swab samples, and each of the eight tissues collected. Only the two ranavirus-inoculated turtles that died due to severity of clinical signs in the 28°C trial had detectable FV3-like DNA. These 28°C-infected turtles were positive in whole blood, cloacal swabs, and all eight necropsy tissues, but not in oral swabs. All control turtles of both temperature trials and the two 28°C-inoculated turtles that failed to develop clinical signs failed to have detectable FV3-like DNA in any clinical or necropsy tissue at any time point.

For the 22°C infected trial there was no significant change over time in the FV3-like copy number in whole blood (P=0.109), oral swabs (p=0.417), or cloacal swabs (p=0.296). There also was no significant difference over time in DNA concentration (whole blood: p=0.259; cloacal swabs: p=0.238; or oral swab samples: p=0.301) or DNA purity (whole blood: p=0.051; oral swabs: p=0.244; cloacal swabs: p=0.330). For the 28°C group there was no significant change over time in the FV3 copy number for any of the samples (whole blood, p=0.153; oral swabs: p=1.00; cloacal swabs: p=0.422), DNA concentration (whole blood: p=0.15; oral swabs: p=0.102; cloacal swabs: p=0.279), or DNA purity (whole blood: p=0.954; oral swabs: p=0.106; cloacal swabs: p=0.197).

The sensitivity and specificity of whole blood was 100% when comparing the terminal sample collected and the presence of ranavirus in necropsy tissues. However, the sensitivity and specificity for oral swabs was 83% and 100%, respectively for the same comparison. However, All infected turtles in the 22°C trial were positive for FV3-like DNA in the terminal sample of whole blood, oral swab, and cloacal swab (Figure 5.7). In the 28°C trial, both infected animals were positive in whole blood in the terminal sample and one each was positive in the terminal samples or oral swab or cloacal swab. The infected animal in the 28°C trial that was negative in

the terminal oral swab sample, was positive in the oral swab sample two days prior. Over the course of the last six sampling points, the infected animal in the 22°C trial that survived the longest (30 days) was positive for detectable FV3-like DNA in oral and cloacal swabs twice, then negative twice in a row, and then positive for the last two samples. The two negative samples corresponded to the lowest whole blood viral copies for that animal.

Clinical signs. Descriptive statistics for clinical signs observed in exposed turtles are presented in Table 5.4. There were no clinical signs recorded that were significantly different between infected and uninfected turtles over the entire study period. However, when evaluating clinical signs between infected and uninfected turtles at the pre, initial post, and terminal time points, there were significant changes over time for nasal discharge ($p=0.018$), ocular discharge ($p=0.018$), lethargy ($p=0.002$), and inoculation site swelling ($p=0.007$). There were no significant differences observed over time in inoculated animals for the presence of oral plaques ($p=0.223$) or skin abscesses ($p=0.135$). There were no observed clinical signs in any control animal or the two 28°C inoculated animals that failed to develop disease at the pre-inoculation, post-inoculation, or terminal time points, except for the observance of lethargy once in one animal and leg swelling twice in two animals. There was no significant change over time in the negative animals for lethargy ($p=0.368$) or leg swelling (0.135).

Pathology. Gross necropsy revealed few changes, but included ulcers of the hard palate in two (25%) animals (Figure 5.2), widening of the nares in four (50%) animals (Figure 5.3), intracoelomic fluid in three (37.5%) animals, and severe disseminated subcutaneous swelling in three (37.5%) animals (Figure 5.4).

The most consistent and significant histologic lesions were observed in the spleens of the 22°C inoculated group and were present in all four (100%) turtles. No changes were detected in

any tissue of any control animal in the 22°C group. Changes included total effacement of the splenic architecture with hemorrhage, fibrin, and heterophils in two of the four turtles. Remaining vessels contained abundant fibrin and heterophils in the vessel wall, and there was lymphoid depletion. The two infected turtles in the 28°C group had similar lesions in the spleen, but architecture was more preserved. The splenic arterioles and ellipsoids were affected, as no vessels observed were spared. One of the control animals in the 28°C group died from severe bacterial septicemia, without the fibrinoid vasculitis observed in ranavirus-infected turtles. Lesions in the liver included thrombi in small vessels and in sinusoids which was observed in all four turtles of the 22°C group. All turtles had hepatocellular intracytoplasmic vacuolar change (glycogen and lipid) and collections of hepatic melanomacrophages; however, there were no differences in magnitude of vacuolar change or melanomacrophage collections between any of the groups of turtles. Vascular lesions of capillary thrombosis were also present in the lung in the 22°C inoculated group, but it was observed in only one (25%) of the turtles. Additionally, the lung of two (50%) out of four turtles in this group had a mild to moderate heterophilic interstitial pneumonia, consisting of heterophils in the faveolar interstitium and increased heterophils in the faveolar capillaries. Two (50%) out of four turtles in this group also had necrotizing myositis, characterized by lytic to degenerating skeletal myocytes that were effaced with fibrin and heterophils. Rare scattered heterophils were present in the liver and ovary of a few of these turtles (Figure 5.2).

Histologic lesions in the 28°C inoculated group were less striking. The two inoculated turtles in this group that were PCR negative for FV3-like virus did not have any significant microscopic lesions. One of the two turtles that were PCR positive for FV3-like virus had intravascular bacilli in multiple vessels that were associated with thrombi, indicating bacteremia

and sepsis. This turtle also had fibrinoid necrosis of the splenic vessels. The other FV3-like PCR positive turtle in this group had a heterophilic interstitial pneumonia and necrotizing myositis, but no lesions were observed in the spleen. Viral inclusions were not observed in any of the turtles from either temperature group. Turtles from all groups had a few granulomas in different organs that were centered on spirorchid-type trematode eggs. Additionally, a few turtles had rare protozoal cysts within skeletal myocytes that were not associated with any necrosis or inflammation. The trematode eggs and protozoal cysts were interpreted as incidental findings.

Eight tissues were collected from each animal at necropsy for FV3-like virus qPCR analysis (Table 5.6). There was a significantly higher viral copy in heart tissue at 22°C compared to 28°C ($p=0.012$), and while there was no significant difference in viral copy found for the other tissues (all $p> 0.05$ for all 63 comparisons), there was a reduction in the number of viral copies found when comparing tissues from animals maintained at 28°C compared to the 22°C (Table 5.5). DNA concentration and purity (A260/A280 ratio) were not significantly different by environmental temperature, and therefore the data was combined ($p>0.05$ for all eight tissue types). Descriptive statistics for DNA concentration and purity are summarized in Table 5.6.

Discussion

Temperature has been determined to have a profound effect on reptile response and progression to many diseases, but has not been described with regard to ranaviral infection. Furthermore, in addition to an anticipated more robust immune response at higher temperatures in reptiles, ranavirus isolates cannot grow above 32°C (Chinchar et al., 2009). These host and pathogen factors would theoretically lead to an increase in survival at higher temperatures.

An increased mortality of red-eared slider turtles exposed to a ranavirus isolate was seen at the lower temperature in this study compared to controls, and a non-significant increase in mortality was seen at the higher temperature. When comparing the mortality between the turtles at the two temperatures, there was no significant difference in mortality but power was lacking in the comparison (Power=0.34). Attempts to strengthen the power of the analysis were made using a larger control population, with the concern that latent disease in the control animals could be problematic. Increased mortality at low temperatures was similarly reported in salamanders (Rojas et al., 2005). Red-eared slider turtles had previously been reported to be susceptible to experimental infection with a ranavirus, but temperature was not controlled in that experiment (Johnson et al., 2007). The temperatures chosen is this study were based in part on the previous experimental challenge simultaneously exposed (access to higher temperature basking spot) to turtles to range of temperatures that included 22°C and 28°C and in part because these temperatures are natural environmental temperatures encountered across the species range in North America (Johnson et al., 2007). While there was a non-significant difference in mortality for red-eared sliders in this study, it may not be similar for other species. However, red-eared sliders are a ubiquitous species in North America and may represent a potential reservoir species. Investigations into the capacity of this species to survive infections at different environmental temperatures with subsequent shedding when environmental temperatures decrease are needed.

There were several clinical signs that were observed in this study. The weight of turtles exposed to ranavirus increased throughout the course of the study in both temperature trials, while there was no significant difference in the weight of the control turtles. Disease processes in most species of mammals, birds, or reptiles can lead to anorexia and subsequent weight loss. However, in this study, the cause of the weight gain may have been due to increased food

consumption or fluid retention. It is unlikely that increased appetite lead to the weight gain because lethargy was a significant finding and turtles were all fed the same amount of diet. In ranavirus-infected turtles, nasal discharge, ocular discharge, lethargy, and inoculation site swelling were also observed. These signs are consistent with other reports in turtles, but are also consistent with general signs of respiratory disease seen with other common pathogens, including *Mycoplasma* and herpesvirus infections (Brown et al. 1994; Christopher at al., 2003; Origgi et al., 2004; Johnson et al., 2005). Another clinical sign of FV3-like infection reported in box turtles and red-eared slider turtles has been oral plaques (DeVoe et al., 2004; Johnson et al., 2007), and while oral plaques on the hard palate were observed in all of the 22°C temperature inoculated turtles, they were not reliably seen over time. The turtles would commonly present with an oral plaque that may be present for several days, but then resolve or result in pinpoint oral ulcers on the hard palate. Regardless, the severity and location (none seen on the tongue) of the oral plaques was not consistent with other cases in box turtles and red-eared slider turtles (Allender et al., 2006; Johnson et al., 2007) or with tortoises with herpesvirus infection (Origgi et al., 2004). One of these previous studies identified a single red-eared slider with an oral plaque on the dorsal palate, which may represent a difference in species pathogenesis. It is possible that while herpesvirus infections may be more chronic and enable plaque development, ranavirus is more acute and development of plaques on the tongue does not occur in this species at these temperatures.

The microscopic lesions of fibrinoid necrosis and vasculitis with thrombosis in multiple organs of infected turtles is consistent with previous studies of ranavirus in turtles (Johnson et al., 2007). This study, like others, also demonstrated that the spleen was the organ that was most frequently and most severely affected histologically. Consistent with the clinical signs and FV3-

like virus PCR findings, turtles in the 22°C infected group all had severe histologic changes within the spleen, and in contrast, the only turtle in the 28°C inoculated group that had splenic lesions was also bacteremic, further supporting the theory that turtles at the lower environmental temperature compared to the higher environmental appear to develop more severe disease. Microscopic lesions in other organs for both groups were inconsistent between turtles. Although a previous paper described splenic lesions centered on splenic ellipsoids and not affecting splenic arterioles, the turtles in this study had lesions in all the splenic vessels. This may reflect the severity of the lesions that were present, potentially obscuring initial lesion in the ellipsoids. It has been proposed that the filtering mechanism of the spleen facilitates the deposition of immune complexes, and therefore the lesions in this organ are a result of virus within circulating inflammatory cells (Johnson et al., 2007).

Viral inclusions were not observed in any of the turtles from either temperature group. Although ranaviral inclusions are a prominent feature of infections in some fish, amphibians, and turtles, other studies have also found that they are not prominent in ranaviral infections in experimentally inoculated red- eared sliders (Johnson et al., 2006). The reasons for the lack of inclusions in these red-eared sliders is unknown, but may be dose dependent, species-specific, temperature-based, or occur during more chronic infections. Future studies should compare several species of chelonians in response to ranavirus infection and varying experimental conditions.

Turtles infected with FV3-like virus and maintained at 22°C survived a median of 24 days and all developed signs of disease, while only two animals in the 28°C trial were euthanized because of the severity of their disease. If the study had been maintained past 30 days, the median survival time for the 28°C group would likely be much longer than was determined in

this study and may have resulted in a significant difference between temperatures. The 30 day study period was chosen for this study based on the hypothesized duration of disease in chelonians with this virus seen in the previous challenge study in red-eared sliders that were conducted over a temperature range that included our temperature trials, but future studies should follow animals past 30 days. Furthermore, there was no DNA evidence of ranavirus in any sample (antemortem or postmortem) in the two inoculated turtles that survived to 30 days in the 28°C group which indicates that FV3-like DNA is either detectable and leads to disease or is undetectable. In cell culture, ranaviruses do not survive above 32°C, and it is likely that as this temperature was approached, the *in vivo* mortality would also decrease, especially in light that reptile immune systems are more active at higher temperatures which are additionally protective (Duguy, 1970).

Quantitative PCR was able to detect ranaviral DNA in every clinical sample type tested in this study in infected animals. While detection in necropsy tissues is invaluable, the usefulness to management of ongoing outbreaks to determine infection in animals prior to death is equally important. The evaluation of the sensitivity and specificity of antemortem sampling of turtles with ranavirus had not been previously evaluated. Common clinical samples previously reported for turtles have been whole blood, oral and cloacal swabs (Allender et al., 2006; Johnson et al., 2007). In the current study, it was determined that whole blood has 100% sensitivity and specificity and would be appropriate clinical samples. However, oral and cloacal swabs had only 83% sensitivity and might therefore lead to false negative results. However, in evaluating the results longitudinally, oral swabs had detectable FV3-like DNA in every infected turtle at some point, while one turtle was negative in cloacal swabs despite being positive in every necropsy tissue. A recent cross-sectional study evaluating ranavirus status in box turtles also demonstrated

that oral swabs and whole blood do not fully agree. In that study, 50% of the positive animals (n=4) were oral swab positive and whole blood negative (Allender et al., 2012b). Different techniques in sampling the oral and cloacal cavity, changes in viral shedding in the oral and cloacal cavities, or changes in systemic viral load make multiple parallel testing strategies needed when performing surveillance studies. Additionally, there may be species differences that lead to differences in viral shedding, or differences in assay specificity in other species due to non-specific inhibitors present in box turtles and not in red-eared sliders.

The viral copies in the clinical samples from the infected turtles quickly went from undetectable to millions or billions of copies. All six diseased turtles had between 2 and 4 positive samples prior to death, corresponding to 1 to 3 weeks duration of viral shedding. This virus appears to associated with an acute disease process in susceptible animals, suggesting there may be limited opportunities to intervene in a clinical setting or when managing an outbreak in a wild population. Furthermore, the disease appears to acutely affect susceptible animals, which may lead to disease events occurring in free-ranging reptiles without being detected. In one turtle during the low temperature trial, the animal developed clinical signs and detectable FV3-like DNA in all clinical samples for 1 week, then whole blood FV3-like DNA decreased for 1 week at the same point that it became undetectable in oral and cloacal swabs, followed by a dramatic increase in viral load in whole blood and reemergence if viral DNA in oral and cloacal swabs. There may be several factors that were responsible for the occurrence in this animal including sampling error, PCR inhibitors, inefficient amplification, but may also reflect a transient host immune response to clear infection. Future studies should investigate lower viral inoculation doses to determine if viral loads fluctuate when the host is not overwhelmed. Furthermore, the acute nature under experimental conditions in this study may not reflect natural conditions and

should be investigated. But if similar, the acute course of disease may limit spread in free-ranging populations of solitary animals, such as the box turtle.

All eight tissue types tested in infected animals had detectable ranavirus, indicating systemic distribution and were non-significantly different from whole blood samples. The highest ranaviral copy numbers were found in skeletal muscle, which is not surprising as this was the site of inoculation. The kidney had the next highest viral copy numbers. The kidney has been proposed as the site of viral persistence in *Xenopus* (Robert et al., 2007). This has not been established for any other species and further work is required to determine if it is true in reptiles. However, there were no observed trends between subjective histopathological severity and viral copy number. Viral loads were higher in all tissues of the 22°C study than the 28°C, but were only significant for the heart and liver.

Turtles in this study were inoculated via intramuscular injection, similar to previous reports in red-eared sliders (Johnson et al., 2007). This is an unlikely mechanism of natural transmission, however vector borne routes (similar to IM injection) have been suggested (Johnson et al., 2007). Natural infections may also occur through direct contact with infected turtles, through water, or via fomites. Transmission through water and direct contact has been documented for amphibians (Brunner et al., 2007). However, oral inoculation of red-eared sliders did not result in infection in a previous report (Johnson et al., 2007). Research is needed to determine the other possible routes of transmission in turtles and the effects temperature has on disease development.

Ranavirus has been suggested as a threat to the biodiversity of amphibian populations (Pearman et al., 2005; Gray et al., 2009a; Teacher et al., 2010; Miller et al., 2011). The threat level on reptile populations is less well-understood, largely due to a lack of epidemiologic and

disease ecologic studies for ranavirus in this group. The red-eared slider turtle has been shown to be a competent model for ranaviral disease, however its ability to adequately translate to other species is unknown. The red-eared slider turtle has an expanding range and likely shares habitat with other animals experiencing ranavirus outbreaks, yet there have been no documented cases in free-ranging red-eared slider turtles to the authors' knowledge. It has been shown that the parasites in the invasive red-eared slider turtles can host-switch to native species outside its traditional range, such as *Emys orbicularis* and *Mauremys leprosa* (Verneau et al., 2011). If the red-eared slider turtle can be a potential host and reservoir, its expanding range may pose a threat to the spread of this virus. Differences in temperature may allow for a reservoir status. When a group of apparently healthy outdoor-raised red-eared sliders were acquired in September and experimentally exposed to 16°C, 42% (n=5) of the animals developed clinical and qPCR evidence of ranaviral disease (unpub. data). The role of the red-eared slider as a reservoir needs further investigation as the expanding range of this species, may likely contribute to outbreaks in novel locations.

In summary, red-eared slider turtles infected with a ranavirus isolate exhibit more mortality and decreased survival time at lower environmental temperatures. Pathological findings of vasculitis are consistent with other reports in turtles. Clinical samples with the highest sensitivity and specificity are whole blood and cloacal swabs and these samples should be collected in suspected cases in the future. Kidney contains the highest viral copy number in systemic samples and is a potential site of primary viral persistence or replication. This study helps to elucidate the epidemiology of ranavirus in chelonians.

Table 5.1. Distribution of weights of red-eared slider turtles over time experimentally exposed to frog virus 3-like virus. The weights, confidence intervals (CI), and minimums/maximums are reported in grams.

		22°C		28°C	
Pre-inoculation	Mean (g)	1693.5625*		1977	
	95% CI	1244.1322	2142.9928	1582.6936	2543.5564
	Min/max	1397.45	2077.50	1654.50	2300.00
Initial post-inoculation	Mean (g)	1692.50*		2005	
	95% CI	1252.99	2132.01	1519.77	2645.23
	Min/max	1390	2060	1606	2404
Terminal	Mean (g)	1802.50*		2097.50	
	95% CI	1310.57	2294.43	1686.49	2632.51
	Min/max	1535	2245	1772	2423

*Significant increase over time ($F=11.1$, $p=0.045$) and significant difference between the change in weights (terminal weight minus the pre-inoculation weight) of infected and uninfected animals ($p=0.035$).

Table 5.2. Descriptive statistics of DNA concentration and DNA purity (A260/A280 ratio) from whole blood (WB), oral swab, and cloacal swab samples collected from red-eared slider turtles after experimental inoculation with frog virus 3-like virus.

			22°C		28°C	
Concentration (μg/ml)						
	WB	Mean	97.35*%		25.31*%	
		95%CI	27.84	166.86	15.48	35.14
		Min/Max	40.1	139.26	17.07	31.98
	Oral swabs	Mean	26.66#%@		12.56#%@	
		95%CI	17.9	35.41	7.3	17.83
		Min/Max	21.06	33.41	9.71	16.24
	Cloacal swabs	Mean	11.39^%@		5.81^%@	
		95%CI	9.11	13.67	3.23	8.38
		Min/Max	9.87	12.92	4.44	7.97
DNA purity (A260/A280 ratio)						
	WB	Mean	1.82		1.81	
		95%CI	1.79	1.85	1.75	1.86
		Min/Max	1.81	1.85	1.77	1.84
	Oral swabs	Mean	1.91		1.83	
		95%CI	1.75	2.08	1.77	1.89
		Min/Max	1.77	2.02	1.79	1.86
	Cloacal swabs	Mean	1.86		1.96	
		95%CI	1.65	2.06	1.71	2.22

	Min/Max	1.68	1.99		1.79	2.17

Table 5.2, continued.

* Statistically significant p=0.044
[#] Statistically significant p=0.005
^ Statistically significant p=0.002
[%] Statistically higher DNA concentration in whole blood than oral swabs (p=0.026) and cloacal swabs (p=0.014)
[@] Statistically higher DNA concentration in oral swabs than cloacal swabs (p=0.001)

Table 5.3. Median viral copy numbers of frog virus 3-like virus that were recovered from red-eared slider turtles experimentally inoculated during transmission studies at 22°C and 28°C. Control turtles all had 0 viral copies at each time point in each temperature group.

		22°C	28°C
Pre-inoculation	Whole Blood	0	0
	Oral swab	0	0
	Cloacal swab	0	0
Initial post-inoculation	Whole Blood	0	0
	Oral swab	0	0
	Cloacal swab	0	0
Terminal	Whole Blood	1.23×10^{7}	6.74×10^{3}
	Oral swab	7.23×10^{6}	$4.23 \times 10^{2\#}$
	Cloacal swab	1.37×10^{6}	$3.36 \times 10^{3\#}$

Only 1 animal had detectable levels

Table 5.4. Proportion of clinical signs observed in red-eared slider turtles experimentally inoculated with frog virus 3-like virus at two environmental temperatures. The proportions represent the presence or absence of clinical signs at any time during the course of the study.

	22°C	28°C
Nasal discharge	4/4	0/2
Ocular discharge	4/4	0/2
Oral plaque	4/4	0/2
Skin abscess	3/4	1/2
Lethargy	4/4	2/2
Leg Swelling	4/4	2/2

Table 5.5. Copies of frog virus 3-like virus detected (per g of tissue) in several necropsy tissues from red-eared slider turtles experimentally inoculated at two environmental temperatures. Median is presented with an *.

		22°C		28°C	
Tongue	Mean/median*	1.25×10^{9}*		5.94×10^{6}*	
	95%CI/10-90% percentiles*	3.58×10^{5}	2.56×10^{10}*	3.58×10^{5}	5.94×10^{6}*
	Min/Max	6.19×10^{5}	3.37×10^{10}	3.58×10^{5}	1.15×10^{7}
Skeletal Muscle	Mean/median*	3.7×10^{10}*		3.64×10^{8}*	
	95%CI/10-90% percentiles*	1.76×10^{10}	1.9×10^{11}*	7.40×10^{7}	3.64×10^{8}*
	Min/Max	1.76×10^{10}	2.39×10^{11}	7.40×10^{7}	6.53×10^{8}
Lung	Mean/median*	6.29×10^{9}*		5.01×10^{9}*	
	95%CI/10-90% percentiles*	3.27×10^{9}	1.94×10^{10}*	2.61×10^{8}	5.01×10^{9}*
	Min/Max	3.27×10^{9}	2.32×10^{10}	2.61×10^{8}	9.76×10^{9}
Heart#	Mean/median*	2.92×10^{10}		1.27×10^{9}*	
	95%CI/10-90% percentiles*	1.58×10^{10}	4.36×10^{10}	7.05×10^{6}	1.27×10^{9}*
	Min/Max	1.89×10^{10}	3.72×10^{10}	7.05×10^{6}	2.54×10^{9}
Liver^	Mean/median*	2.15×10^{9}		1.70×10^{7}*	
	95%CI/10-90% percentiles*	1.13×10^{9}	3.16×10^{9}	1.87×10^{6}	1.70×10^{7}*
	Min/Max	1.32×10^{9}	2.85×10^{9}	1.87×10^{6}	3.21×10^{7}

Table 5.5, continued

Spleen	Mean/median*	2.23×10^{10}*		5.44×10^{7}*	
	95%CI/10-90% percentiles*	6.43×10^{9}	1.4×10^{11}	1.52×10^{8}	5.44×10^{8}*
	Min/Max	6.43×10^{9}	1.77×10^{11}	1.52×10^{8}	9.36×10^{8}
Ovary	Mean/median*	8.93×10^{9}*		9.06×10^{6}*	
	95%CI/10-90% percentiles*	4.53×10^{8}	1.28×10^{10}*	0	9.06×10^{6}*
	Min/Max	4.52×10^{8}	1.31×10^{10}	0	1.81×10^{7}
Kidney	Mean/median*	3.46×10^{10}*		2.54×10^{8}*	
	95%CI/10-90% percentiles*	1.77×10^{9}	5.16×10^{10}*	2.43×10^{7}	2.54×10^{8}*
	Min/Max	1.77×10^{9}	5.69×10^{10}	2.43×10^{7}	4.84×10^{8}

[#] Significant difference between environmental temperatures, p=0.012
^ Significant difference between environmental temperatures, p=0.011

Table 5.6. Descriptive statistics for DNA concentration and purity (A260/A280 ratio) from extracts of tissues taken at necropsy from red-eared slider turtles experimentally infected with frog virus 3-like virus performed at two environmental temperatures.

	Mean	95% Confidence Interval		Min/Max	
DNA concentration (ug/ml)					
Tongue	139.90	11.20	268.60	2.95	278.40
Lung*	107.57	20.56	194.58	24.88	241.17
Heart^	38.24	15.92	60.55	3.04	65.96
Liver	151.78	41.47	262.08	63.62	351.62
Spleen	97.18	33.26	161.10	12.01	170.79
Skeletal Muscle#	23.85	7.80	39.90	6.65	40.03
Ovary	71.06	24.84	117.29	3.38	134.89
Kidney	109.99	26.20	193.78	.96	229.96
DNA purity (A260/A280 ratio)					
Tongue	2.61	.71	4.50	1.89	5.34
Lung	1.84	1.75	1.92	1.69	1.89
Heart	1.72	1.46	1.98	1.25	1.91
Liver	1.70	1.53	1.86	1.39	1.83

Table 5.6, continued.

Spleen	1.86	1.78	1.93	1.72	1.93
Skeletal Muscle	1.72	1.54	1.90	1.43	1.93
Ovary	1.79	1.55	2.02	1.33	1.93
Kidney	3.80	0	8.73	1.86	13.40

* Significantly higher than skeletal muscle, p=0.046
^ Significantly lower than liver (p=0.025), spleen (p=0.028), ovary (p=0.046), and kidney (p=0.046)
# Significantly lower than liver (p=0.033), spleen (p=0.035), ovary (p=0.031), and kidney (p=0.031).

Figure 5.1. Survival analysis of red-eared slider turtles experimentally exposed to frog virus 3-like virus at 22°C (1; blue) and 28°C (2; green). State what the temperatures are for each legend.

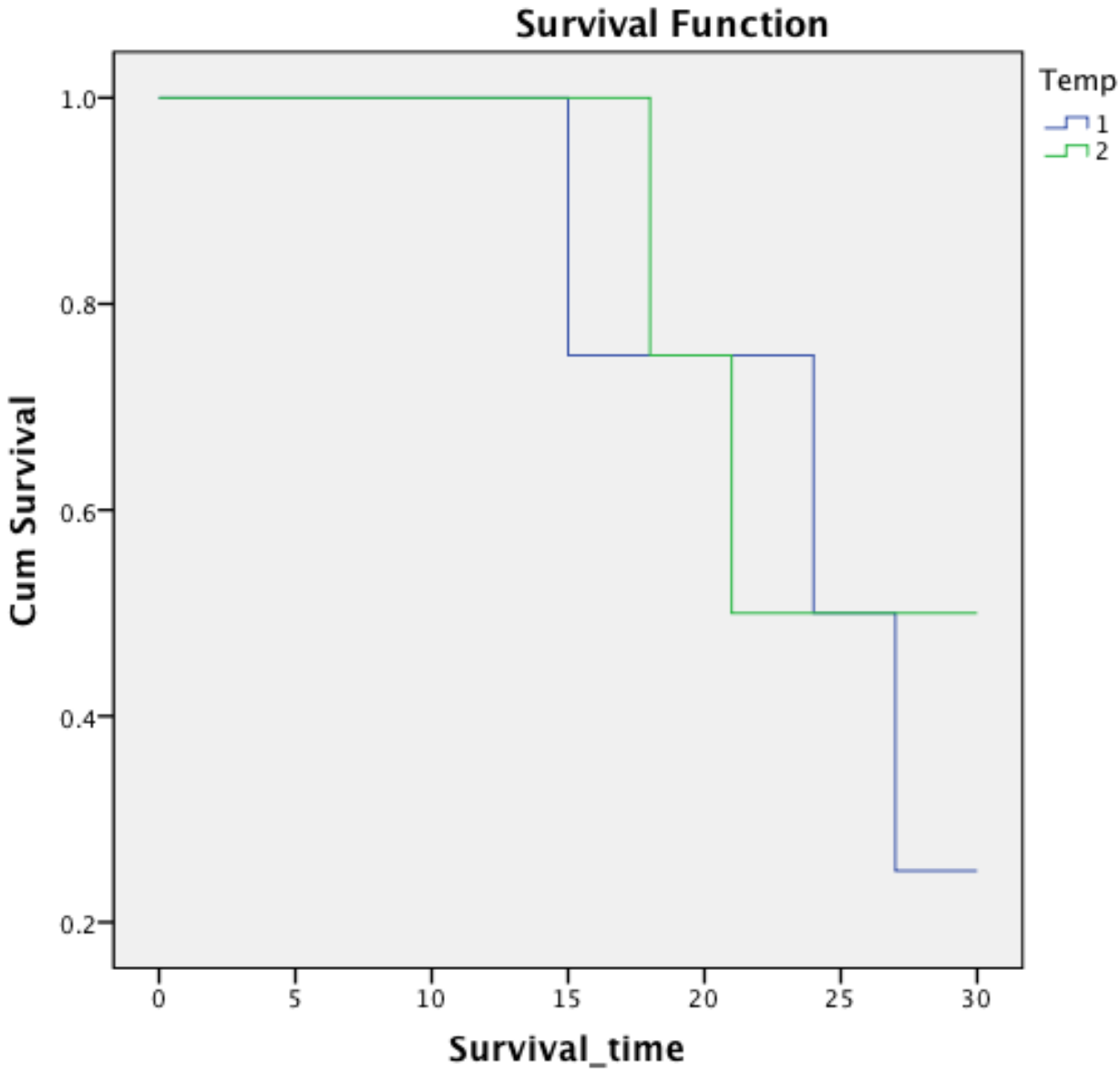


Figure 5.2. Oral plaque on the dorsal palate of the oral cavity of a red-eared slider experimentally inoculated with ranavirus.

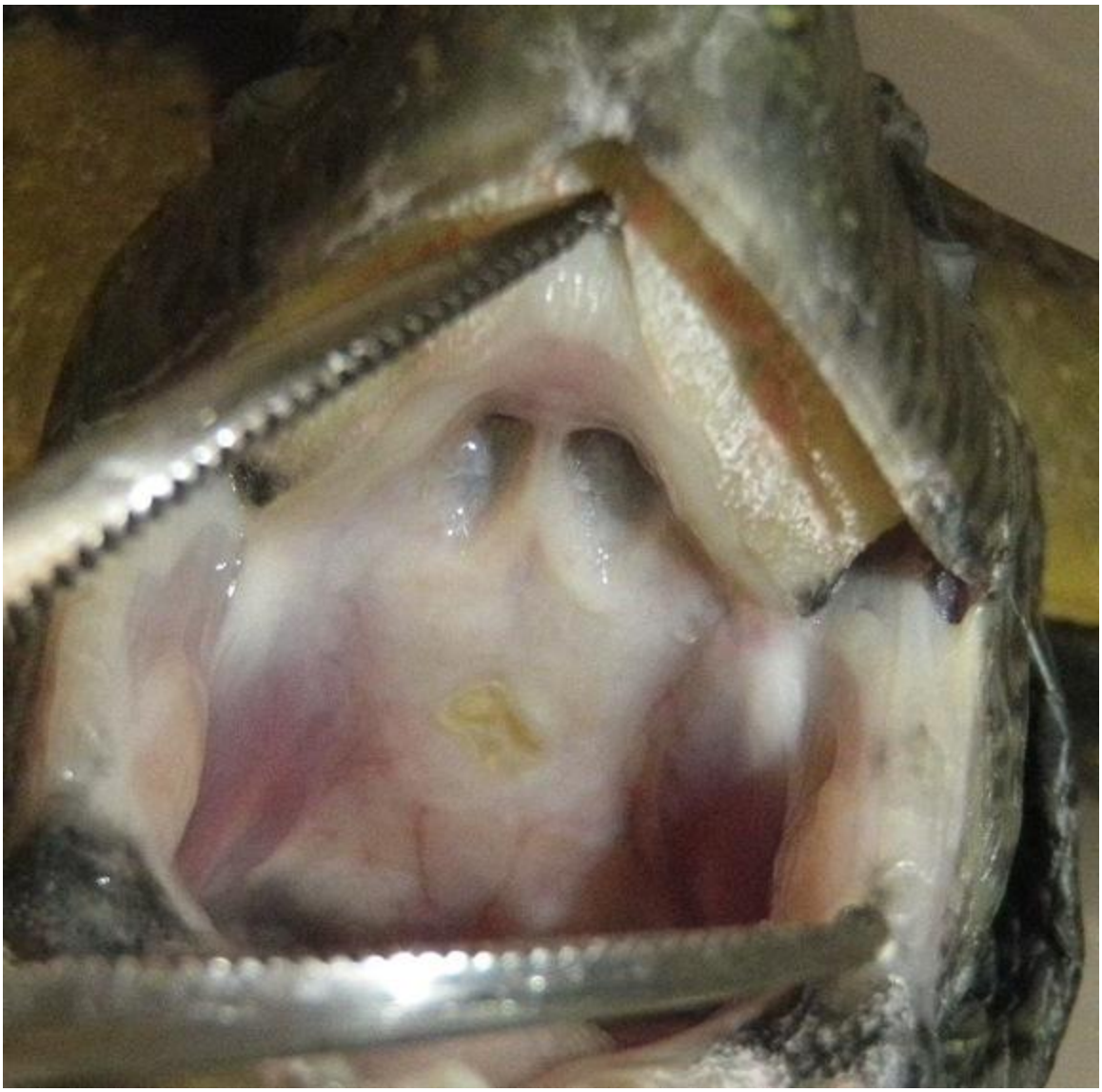


Figure 5.3. Oral ulceration in a red-eared slider after experimentally inoculated with ranavirus.

Figure 5.4. Widening of the nares observed in a red-eared slider experimentally inoculated with ranavirus.

Figure 5.5. Ranavirus-infected red-eared slider demonstrating severe edema that occurred in less than a 24 hour period at 28C. The animal was also confirmed to be septic.

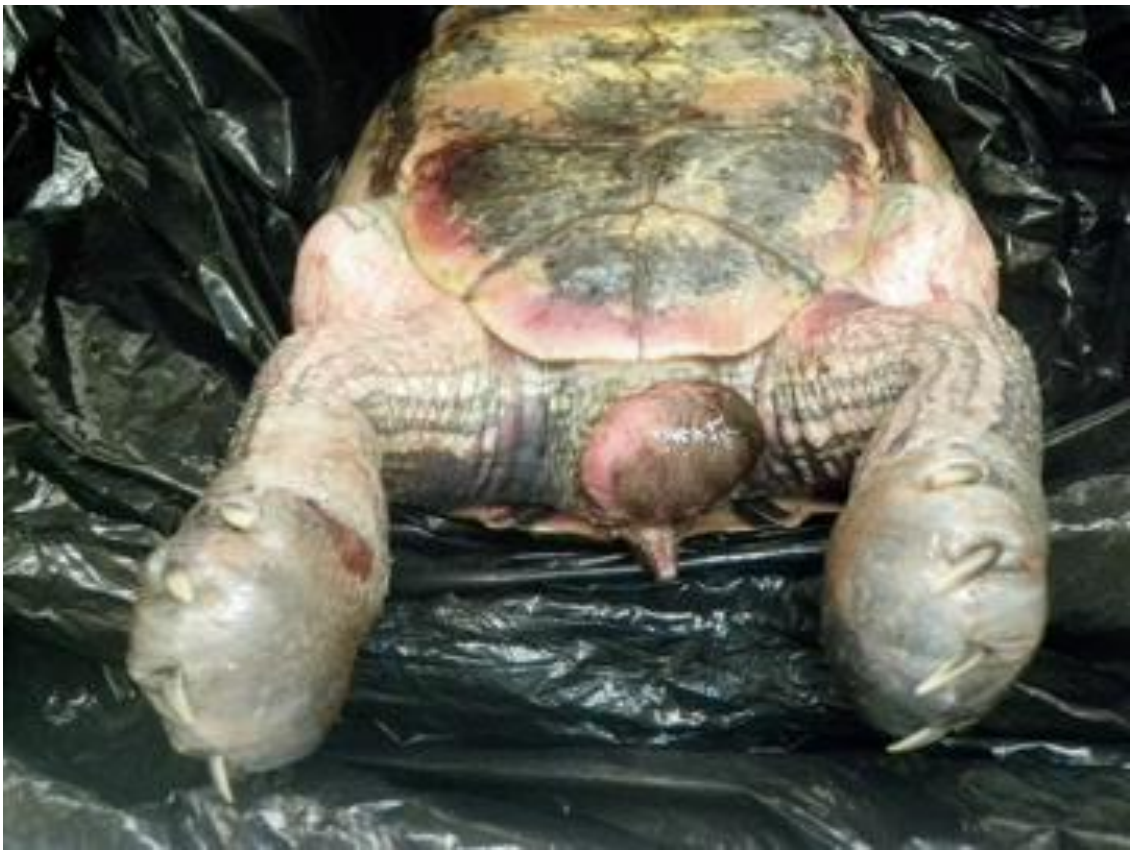


Figure 5.6. (A) Red eared slider in 22°C ranavirus-challenge group with no lesions in the spleen. H&E. (B) Red eared slider in 22°C inoculation group with splenic necrosis that effaces the splenic architecture. H&E. (C) Red eared slider in 22°C inoculation group with fibrinoid necrosis of a splenic vessel. H&E. (D) Red eared slider in 22°C inoculation group with heterophilic interstitial pneumonia. Heterophils fill faveolar capillaries and are present in the interstitium (asterisk). H&E. (E) Red eared slider in 22°C inoculation group with a thrombus filling a hepatic vessel. H&E. (F) Red eared slider in 22°C inoculation group with thrombi in hepatic sinusoids (arrows). H&E. (G) Red eared slider in 22°C transmission group with no lesions in the skeletal muscle. H&E. (H) Red eared slider in 22°C inoculation group with necrotizing myositis. Fibrin and heterophils efface the skeletal myocytes and myocytes exhibit necrosis and degeneration. H&E.

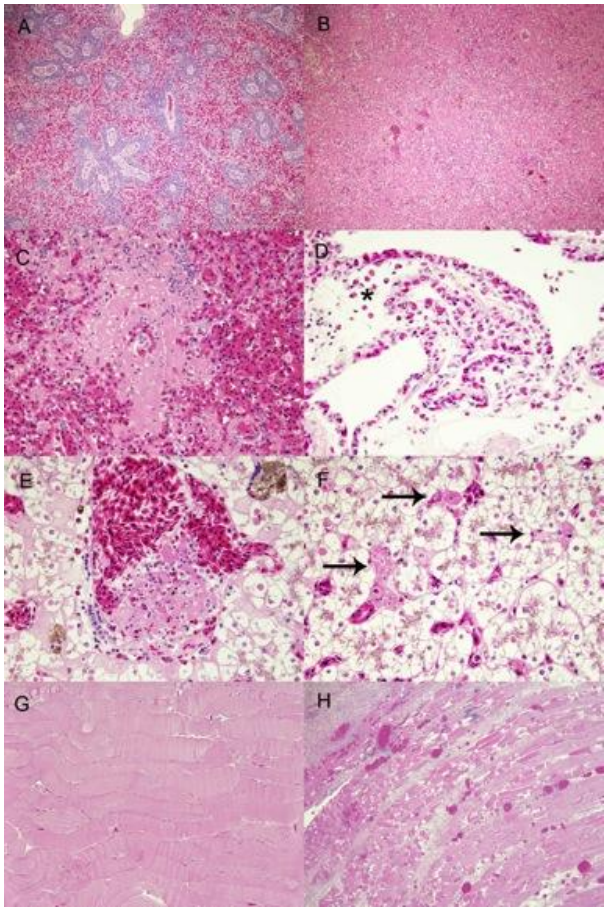
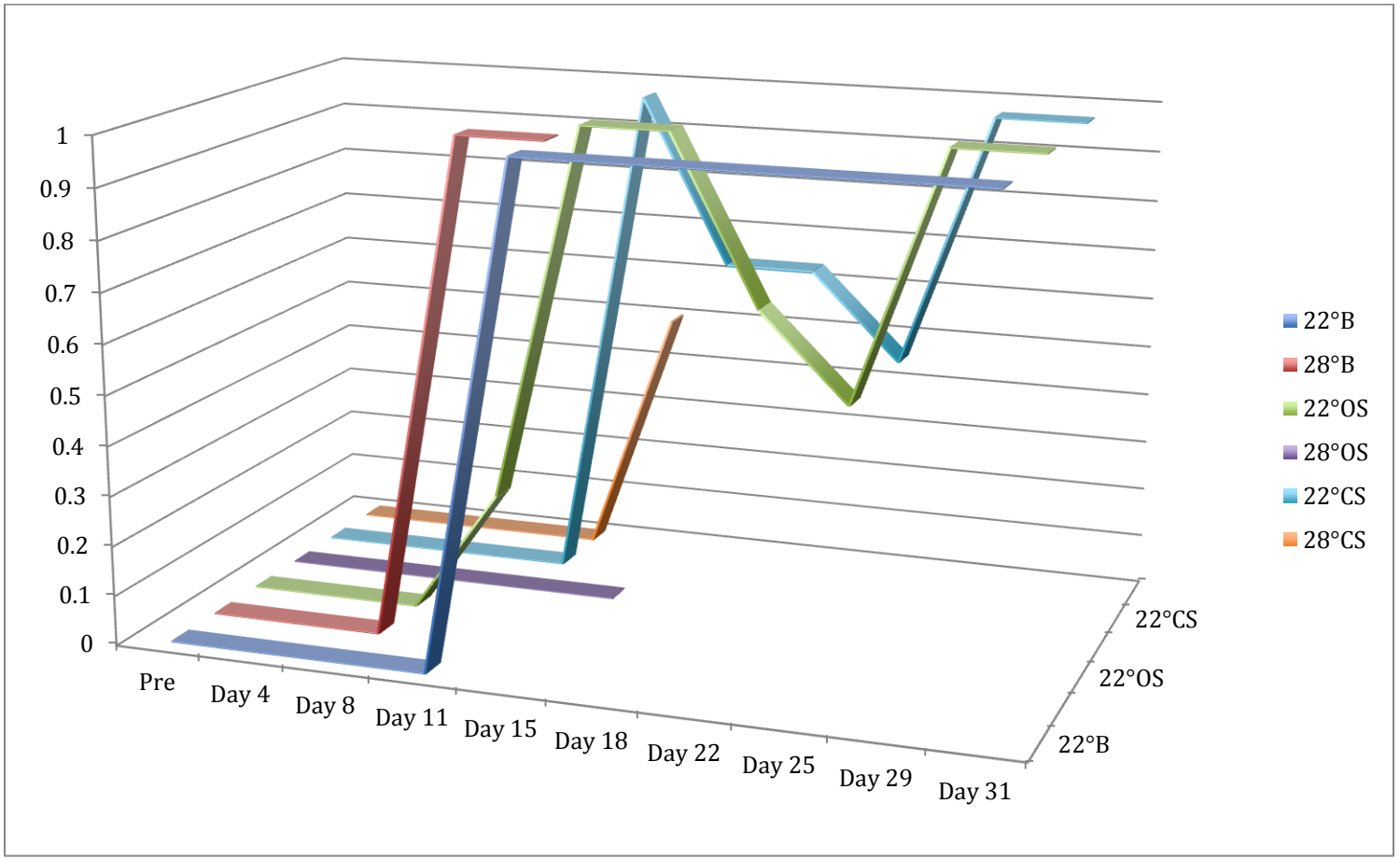


Figure 5.7. Proportion of frog virus 3-like DNA detected whole blood (B), oral swabs (OS), and cloacal swabs (CS) 5/6in red-eared slider turtles at ten time points before and after experimental inoculation in eight red-eared sliders at 22°C and 28°C.



CHAPTER 6

HEMATOLOGIC RESPONSE TO EXPERIMENTAL AND NATURAL INFECTIONS OF FROG VIRUS 3-like virus IN TWO SPECIES OF CHELONIANS[4]

Abstract: Clinical pathology is routinely used to diagnose and monitor response to disease. Hematologic changes during ranavirus infection have yet to be characterized. In this study, hematology was analyzed in sixteen red-eared slider turtles (*Trachemys scripta elegans*; RES) and 384 eastern box turtles (*Terrapene carolina carolina*; EBT). Six red-eared slider turtles and three box turtles were infected experimentally or naturally with a ranavirus, frog virus 3-like virus (FV3-like), respectively. Total solids were significantly decreased (p=0.034) over time in the red-eared slider turtles infected with FV3-like virus. Lymphocytes were significantly decreased in box turtles infected with FV3-like virus, and a non-significant decreasing trend in lymphocyte numbers was noted in red-eared sliders with FV3-like infection. Intracytoplasmic inclusions were seen in variable prevalence in box turtles in different institutions (0 – 44%). Presence of inclusions in WBC were observed in non-FV3-like infected EBT, but were not seen in uninfected RES.. Inclusions were seen in two red-eared slider turtles and one box turtle with FV3-infection. White blood cell count was higher in box turtles with inclusions, regardless of FV3 status. Hematology may be a valuable tool for monitoring disease in turtles with FV3-like virus, but further work is needed with higher sample sizes to characterize the actual response.

Key Words: Reptile, *Terrapene*, *Trachemys*, hematology, iridovirus

[4] Content of this chapter has been partially or fully submitted for publication as cited in bibliography as Allender et al., 2012d.

Introduction

Several terrestrial and aquatic chelonians are experiencing population declines across the world. The International Union for Conservation of Nature 2011 redlist currently considers 32 chelonians as critically endangered, 44 as endangered, and 58 as vulnerable. It lists an additional species as conservation dependent and 36 as near threatened (van Dijk, 2011). Therefore, of the 328 total species of chelonians in the world, 178 (53%) are listed with population stability as near-threatened or worse, including 8 species that are already extinct (Rhodin et al., 2010). The North American box turtle (*Terrapene carolina carolina*) has recently experienced significant population declines in numerous areas throughout its range, leading to its population status being downgraded in 2011 from near threatened to vulnerable (van Dijk, 2011). Free-ranging box turtles have experienced numerous disease events that may impact population stability, including ranavirus infections (Sleeman, 2008, Donaldson and Echternact, 2005; Ultsch, 2006; Allender et al., 2012b).

Ranaviruses are a significant threat to populations of amphibians and reptiles (Miller et al., 2011). Diagnostic assays that can quickly and correctly identify the presence of ranavirus are crucial to early diagnosis and potential intervention. Currently, quantitative PCR (qPCR) or conventional PCR, viral isolation, and histopathology are the primary diagnostic methods used to confirm the presence of this pathogen (Allender et al., 2012a; Johnson et al., 2007). However, a previous report has described the presence of intracytoplasmic inclusions within circulating white blood cells with ranavirus, specifically frog virus 3-like virus (FV3-like) infection (Allender et al., 2006). Differential white blood cell counts are often used as a non-invasive field health assessment tool. The advantages of this type of diagnostic test are that it can be done in the field, having a more rapid turnaround time than qPCR, and is not as invasive as biopsy and

histopathology. These characteristics might make evaluating the relationship and progression of complete blood cell count parameters ideal for characterizing or managing outbreaks in vulnerable species.

Intracytoplasmic inclusions have rarely been associated with white blood cells in reptiles, except for a case report of ranavirus in a box turtle (Allender et al., 2006) and a report of *Chlamydia* and poxvirus in monocytes of chameleons (Jacobson and Telford, 1990). More often, inclusions within erythrocytes have been reported in reptiles due to viruses, parasites, and degenerate cellular components (Alleman et al., 1992; Telford and Jacobson, 1993; Clark et al., 2001; Basile et al., 2011; Wellehan et al., 2008; Alves de Matos et al., 2002; Johnsrude et al., 1997). While two of these erythrocyte reports involve iridoviruses, ranavirus-specific inclusions have not been reported. Other tissues are more commonly observed to develop inclusions, most notably associated with viruses such as herpesviruses, adenoviruses, and inclusion body disease of boids (Rivera et al., 2009; Jacobson et al., 1986; Wozniak et al., 2000).

The specific aim of the study was to evaluate the hematological response of two species of chelonians to ranavirus infections under experimental and natural infections. Additionally, a secondary aim was to determine if inclusions within white blood cells occur routinely as part of this response. The biological hypotheses tested in this study were: 1) There will be no difference in complete blood count (CBC) parameters [packed cell volume (PCV), total solids (TS), total white blood cell count (WBC), heterophils, lymphocytes, monocytes, eosinophils, or basophils] after experimental FV3-like infection in red-eared slider turtles (RES) (*Trachemys scripta elegans*); 2) Intracytoplasmic inclusions will be found within white blood cells of red-eared sliders experimentally infected with FV3-like virus; 3) There will be no difference in CBC parameters between naturally occurring FV3-like positive and FV3-like negative eastern box

turtles (EBT) (*Terrapene carolina carolina*); and 4) Intracytoplasmic inclusions will be will be found within white blood cells of box turtles naturally infected with FV3.

Materials and Methods

Experimental study. All activities were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol: 11050). Animals in this study were concurrently sampled as part of a separate study (Allender et al., 2012c). Sixteen adult female RES were acquired from a commercial turtle farm. Using computer-generated randomization, four animals were assigned to receive inoculation and four animals were assigned to remain as uninfected controls in each of the two environmental temperature trials. The first trial was carried out in an 15'9" x 12" environmental chamber held at 22°C. This trial was carried out with both the inoculated and control animals in the same room separated by a double plastic barrier and 2-foot hallway created between the plastic barriers. The second trial was carried out with the inoculated and control animals housed in separate, but identical, environmental chambers at 28°C. Each trial was terminated at 31 days post-inoculation. The first trial began in July 2011 and the second began in November 2011. Animals were housed singly in 45 or 50 gallon plastic enclosures with 20 gallons of water and access to a dry dock (cinder block). Animals were fed a commercial aquatic turtle diet (Fluker Farms, Port Allen, LA) every other day.

A *Ranavirus* isolate was grown in *Terrapene* heart cells (TH-1). Flasks were scraped when cells exhibited 100% cytopathic effects. Cells and media were then transferred to 15ml tubes, centrifuged at 4,500x*g* for 30 minutes, and the supernatant discarded. The cell pellets were resuspended in residual media, then frozen and thawed three times, thoroughly vortexed before and after each freeze cycle, and centrifuged again at 4,500x*g* for 30 minutes. Quantitative PCR

was then performed to confirm the presence and quantify viral DNA. Viral titers were determined using serial dilutions of virus in cell culture.

At the start of the trial each animal was examined and weighed; blood (1.5 mls), oral and cloacal swabs (plastic handled cotton-tipped applicator, Fisher Scientific) were collected 7 days (-7 d) and 4 days (-4 d) before the virus challenge. Blood samples were collected from the subcarapacial vein. Quantitative PCR was performed on the whole blood and both swabs from both days to confirm negative status. On day 0, each treatment animal was given 5×10^5 $TCID_{50}$ FV3-like virus intramuscularly in the right forelimb. Each control animal was administered an equal volume of uninfected TH-1 cell lysate on the same day. Each animal was weighed and clinical samples (blood, oral and cloacal swabs) were collected from each animal on days 3, 7, 10, 14, 17, 21, 24, 28, and 31 post-inoculation. Whole blood was immediately placed in two lithium heparin microtainers, while the swabs were each placed in separate 2.0 ml polypropylene eppendorf tubes. All samples were stored at -20°C until analysis.

Natural Infection. All procedures were approved by the Institutional Animal Care and Use Committees at the University of Tennessee (UT: 1630-0507) and University of Illinois (UI: 10057). The box turtles evaluated in this study were subjects from a concurrent study (Allender et al., 2012b) that had multiple objectives. The study population included eastern box turtles presented to wildlife centers at the University of Tennessee (UT)(Knoxville, TN), Wildlife Center of Virginia (WCV)(Waynesboro, VA), and North Carolina State University (NCSU) (Raleigh, NC) in 2007(Allender et al., 2011; Allender et al., 2012a; Allender et al., 2012b); and from free-ranging, wild box turtles in Oak Ridge, TN (OR) in 2010 and 2011 (Way and Allender, 2012).

Blood samples were collected from the subcarapacial sinus and immediately placed into a lithium heparin coated microtainer (Becton Dickinson, Franklin Lakes, NJ). The blood tubes were labeled with a unique identification number and remained on-site at -20ºC, batch shipped, and stored at -20ºC until analysis. Samples were also processed to create two blood smears, stained with diff-quik (Dipquick, VetLab Supply), and shipped with whole blood tubes. Slides of blood smears collected in 2007 were cover-slipped for optimal preservation, while slides from 2010 and 2011 were evaluated at the time they were made.

Quantitative PCR. Quantitative PCR was performed as previously described (Allender et al., 2012a). Briefly, DNA was extracted from whole blood using the manufacturer's instructions (QIAmp Blood Mini Kit, Qiagen, Valencia, CA). TaqMan assay was performed using forward (AACGCCGACCGAAAACTG), reverse (GCTGCCAAGATGTCGGGTAA), and probe (CCGGCTTTCGGGC) targeting a 54 bp segment of the major capsid protein (MCP) of FV3. All samples were assayed in three technical repeats using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA), analyzed using commercial software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, CA), and results averaged. Quantitative PCR results for 2007 UT samples (Allender et al., 2012b) and 2007 WCV, 2007 NSCU, and 2010-2011 OR samples that had separate objectives have been presented previously (Allender et al., 2012b), but are referenced in this study only as it pertains to the effect of FV3 infection on clinical pathology results and leukocytic inclusions.

Complete Blood Count (CBC). For the experimental study and the OR animal samples collected in 2010 and 2011, whole blood was placed in two microhematocrit tubes, centrifuged, and percent red cells manually determined (PCV). Microhematocrit tubes were broken at the interface of red cells and plasma, plasma placed on a refractometer, and total solids recorded

(TS). An avian leukopet system (VetLab Supply, Palmetto Bay, FL) was used to stain heterophils and eosinophils according to the manufacturer's instructions and counted routinely on a hemacytometer. Differential white blood cell counts were manually evaluated using light microscopy with 40X objective. Percent heterophils and eosinophils were used with the results of the hemacytometer to determine total white blood cell (WBC) counts as previously described (Campbell, 2006). For all of the box turtles presented to rehabilitation centers, estimated white blood cell counts were performed from a stained blood smear. Differential cell counts were performed by several individuals, but all individuals were trained similarly and a subset of slides from each year were confirmed by the primary author (MCA). Presence of intracytoplasmic inclusions within white blood cells was recorded as present or absent for each sample. A minimum 100 white blood cells or 100 fields (under 40X objective) were evaluated. Inclusions were identified based on size, shape, color, and displacement of the nucleus. CBC results from 2010 OR have previously been reported with independent objectives (Way and Allender, 2012), but were referenced and combined with 2011 data (metanalysis) in this study only as it pertains to the effect of FV3 infection on clinical pathology and leukocytic inclusions.

Statistical analysis. Descriptive statistics were produced for each continuous variable (weight, WBC, PCV, differential white blood cells, TS) for each time point, including the mean/median, 95% confidence interval/10-90% percentiles, and minimum/maximum. Normality of data was determined using the Shapiro-Wilk test. In the experimental study, due to missing values from individuals that died due to the study design, four time points were included in the final analysis: the "pre-" sample (two pre-inoculation samples were averaged), the immediate "post-"inoculation sample, and the "terminal" sample (the last sample taken prior to death). The timing of the pre- and the post-samples were the same days for every RES; however, the final sample

was collected at the last time point prior to animal death (day 7- 31 post-inoculation). Repeated measures (Friedman's for non-normally distributed data or repeated measures ANOVA for normally distributed continuous variables described above) analysis was performed over time, independent of treatment and temperature exposure in RES. Then, separately, the same analysis was performed for turtles in each temperature and RES treatment group, respectively. Specific between group (temperature and exposure) differences for RES PCV, TS, and WBC were further evaluated using a paired sample t-test/related samples Wilcoxon signed rank test. Specific group (institution, sex, age, year) differences for WBC in EBT were evaluated using Kruskal-Wallis one-way ANOVA and a Mann-Whitney U for between group differences. Differences between temperatures for continuous variables at the same time point in RES were evaluated using an independent samples t-test. Presence or absence of inclusions within white blood cells was tabulated (categorical variable) and compared based on temperature (RES), sex (EBT), age (EBT), location (EBT), and FV3-like-status (all). Statistical significance was considered for all p values <0.05. All analysis was performed using SPSS 20 statistical software (IBM Statistics, Chicago, IL).

Results

RES. Packed cell volume, total solids, and total WBC over each sample period are shown for each temperature in Table 6.1. When evaluating the pre-inoculation, post-inoculation, and terminal samples for RES, there was no significant difference in any CBC parameter in the challenged turtles between the two environmental temperatures and therefore the data was combined (Table 6.2). There was no difference between pre-inoculation, post-inoculation, and terminal samples for PCV ($p=0.444$) or WBC ($p=0.421$) for infected turtles. There was a

significant decrease over those three time points for total solids ($p=0.034$) in infected turtles. For white blood cell differentials, heterophils ($p=0.236$), lymphocytes ($p=0.862$), monocytes ($p=0.325$), eosinophils ($p=0.497$), and basophils (0.165) were not different over time in infected turtles. Intracytoplasmic inclusions were seen in two infected animals in the last sample that was collected prior to death. There were no differences in prevalence of intracytoplasmic inclusion between infected and uninfected turtles.

Eastern box turtles. White blood cell counts were performed in 84 turtles in 2007, 170 in 2010, and 134 in 2011. Differential cell counts were performed on 48 turtles in 2007, 170 in 2010, and 134 in 2011. There was a significant difference in WBC ($p<0.0001$), heterophils ($p<0.0001$), lymphocytes ($p<0.0001$), monocytes ($p<0.0001$), eosinophils ($p<0.0001$), and basophils ($p<0.0001$) based on method of quantification. There was a significant difference in WBC ($p<0.0001$), heterophils ($p<0.0001$), lymphocytes ($p<0.0001$), monocytes ($p<0.0001$), eosinophils ($p<0.0001$), basophils ($p<0.0001$), PCV ($p<0.0001$), and TS ($p=0.041$) by year (Table 6.3 and Table 6.4). There was no significant difference in weight by year ($p=0.141$). White blood cell inclusions were seen only from rehabilitation turtles in 2007 (Figure 6.1). White blood cell inclusions were seen with significantly different proportions based on institution ($p<0.0001$). White blood cell count in turtles with (median 11,200 cells/µl; 10-90% percentile: 1640 – 21880 cells/µl) inclusions was non-significantly higher than without (5200 cells/µl; 10-90% percentile: 3800 – 46000 cells/µl). White blood cell count was significantly higher for turtles in 2007 that had inclusions (Table 6). There was no association between FV3-like virus positive animals and white blood cell inclusions ($p=0.307$). The third positive animal in 2007 did not have a blood smear evaluated. The two FV3-like virus positive animals that had estimated WBC had counts of 14400 and 11600 cells/µl, which was not significantly different from FV3-

like virus negative turtles (p=0.147). The predominant white blood cell in both FV3-like infected box turtles was the heterophil 5904 and 10018 heterophils/µl, respectively, which was not significantly different from FV3-like virus negative turtles (p=0.858). Neither of the FV3-like virus positive turtles had any lymphocytes which was significantly lower than FV3-like virus negative turtles (p=0.044)

Discussion

Hematology is a commonly used diagnostic assay to evaluate the general health and disease status of humans and animals. While this diagnostic is also commonly used with reptiles (Campbell, 2006), reference intervals for many species of reptiles have not been established, and when established can vary by season, potentially limiting the value of this diagnostic method. Furthermore, the hematologic response following exposure to specific pathogens is unknown in reptile diseases, including ranavirus. The characterization of this response could be invaluable in the diagnosis and therapeutic monitoring of diseases in reptiles and is worth pursuing.

This study monitored the hematological response in two species of chelonians to an emerging viral disease, FV3, under experimental and natural conditions. In the case of experimental inoculation of FV3-like virus in red-eared sliders, the only variable that was significantly different over time was a decrease in total solids (44.7%). Total solids as determined by refractometer are a measure of the total protein in the body. Low protein may be seen with malnutrition, severe blood loss, or chronic liver or kidney disease (Campbell, 2006). These animals had significant vasculitis has a hallmark of the pathologic response, which may have accounted for leakage of proteins from the vasculature (Allender et al., 2012c). Additionally, multi-systemic organ failure is commonly seen as the cause of death in ranavirus

infections in chelonians and may account for the hypoproteinemia. It is notable that a viral infection would be expected to cause a response in humoral immunity that would cause an increase in total solids, but the acute nature of ranaviral disease likely doesn't allow an individual to mount a response. Future studies need to look at protein electrophoresis and acute phase proteins, which are currently being pursued.

The WBC is commonly used to diagnose or monitor the progression of an inflammatory response in a vertebrate. In this study, the WBC was not significantly different in RES or box turtles with FV3-like infection compared with conspecifics without disease. The absence of a change in the WBC could have been due to the natural response of the host to this particular virus or the virus' ability to elude the host defense mechanisms or study related factors such as a small sample size and a limited study duration. Season has been shown to cause significant variation in WBC in reptiles (Duguy, 1970), and may have a profound impact on the response seen to infectious pathogens. There were too few FV3-like virus positive samples in this study to investigate season, but ongoing monitoring is being performed in a free-ranging population. White blood cell counts were significantly different by year in eastern box turtles. This difference may be due to time of year sampled, potential exposure to infectious disease, or subjective evaluation of blood smears. There was no significant difference between 2007 (blood smear estimate) and 2011 (leukopet) despite different methods of determining the WBC. However, the leukopet method used in 2010 and 2011 was the same, possibly indicating that the person evaluating the differential white blood cell count might be a confounding factor. Each year had a separate individual evaluating the blood smears and might introduce variation into the evaluation.

Differential WBC types were not significantly different between FV3-like virus positive and FV3-like virus negative turtles for any cell type in either study, except for a decrease in lymphocytes seen in box turtles with FV3-like infection. There was also a trend towards a reduction in the lymphocytes in the RES, but it was not significant (observed power ranged from 0.05 through 0.448). Lymphocytes are commonly increased in response to viral disease to modulate cell-mediate immunity in mammals. In acute diseases or in early stages of a disease process, lymphocytes may be mobilized to the sites of viral infection prior to the compensatory increased production. This might explain why there was a decrease in lymphocytes in our study. Increased sample size would be needed to determine if lymphopenia is a feature of ranavirus infection in turtles. Low observed power was determined post-hoc indicating a potential Type II error. The response to specific pathogens in tortoises is largely anecdotal, however tortoises with herpesvirus infection demonstrated lymphocytosis and heteropenia compared to uninfected animals (Muro et al., 1998). This difference in lymphocyte proportion between herpesvirus and ranavirus might be due to the course of disease. Herpesvirus can be more chronic in tortoises (Origgi et al., 2004), thereby allowing ample time for subsequent response to viral infection. Future studies should critically evaluate lymphopenia in turtles demonstrating clinical evidence of ranavirus, and may be helpful in documenting clinical improvement.

Packed cell volume, a measure for red blood cell concentration, is commonly used to evaluate animals for the presence or absence of anemia. The PCV reference interval calculated for these animals was consistent with other studies. Interestingly, the PCV values obtained from these turtles were not significantly different based on FV3-like status in either the experimentally infected RES or naturally infected box turtles. Anemia is commonly seen is chronic disease states in vertebrates, and reptiles are no exception (Campbell, 2006), but is not routinely reported

in reptiles. It is possible that no change was noted because of the acute nature of the ranavirus infection. The longevity of erythrocytes in reptiles is much longer than in mammals, with some snakes having erythrocytes live for as long as 600 days (Campbell, 2006). Therefore, the cell loss may not be detected in a disease of short duration under natural infection or in the experimental study presented here because it was limited to 30 days. Additionally, pure blood samples in reptiles are commonly diluted with lymph fluid due to the confluence of blood and lymph vessels in close approximation, including the subcarapacial sinus. While the jugular vein may be the preferred site for venipuncture to minimize the likelihood of sample dilution, it is often not possible to collect samples from this site in chelonians without anesthesia. In the turtle sample populations presented here, there were some PCV values that were considered low, and likely diluted with lymph. However, it appears that the potential for dilution was similar across the different turtle groups.

Inclusions in tissues are commonly seen with many viruses (Johnson et al., 2007), and inclusions in circulating white blood cells have been reported in a box turtle with ranavirus (Allender et al., 2006). In this study, we determined that inclusions in the white blood cells occur with greater frequency in box turtles than red-eared sliders, and may be high in turtles without qPCR detectable ranavirus. This study found a high prevalence in rehabilitation turtles that presented to one institution. Potential causes of these cytoplasmic inclusions need to be investigated, but may include other viruses, parasites, and active or degenerate cellular components (Alleman et al., 1992; Telford and Jacobson, 1993; Clark et al., 2001; Basile et al., 2011; Wellehan et al., 2008; Alves de Matos et al., 2002; Johnsrude et al., 1997). Turtles in this study had evidence of active inflammation with cellular granules (basophilic stippling) that may be mistaken for inclusions. Basophilic stippling has been reported previously in reptiles in

response to inflammation (Campbell, 2006). However, inclusions were characterized in this study based on cellular type, size, shape, and relationship to nucleus. Electron microscopy, such as that performed to confirm a previous report of ranavirus will be required in future cases and should be pursued.

There were several limitations identified in this study. First, the two methods of determining the total white blood cell count introduced bias because one method relied on the subjective evaluation of a blood smear. Attempts to minimize this bias were to have the same individual (MCA) train all individuals and evaluate a subset of slides from all years and locations. However, it is still possible that this lead to the significant differences observed. Additionally, the method of determining inclusions was not specific and needs to be expanded to include electron microscopy. And lastly, total solids were determined using a refractometer, and more accurate determination using protein electrophoresis needs to be performed.

In summary, the acute nature of ranavirus infection in chelonians does not appear to result in significant changes in a hematologic profile in these animals. Furthermore, while intracytoplasmic inclusions are a characteristic of ranavirus infection in turtles, this is neither a feature consistently observed nor a finding specific to ranavirus. Hematology may be a valuable tool for monitoring disease in reptiles, but further work is needed with higher sample sizes of FV3-like virus positive turtles to characterize the actual response. Equipment that is capable of minimizing the potential bias used with the techniques currently used to process these samples is currently not available, but may soon be. If it is available, utilizing it may prove useful. In addition, research should focus on other areas that evaluate the immune response including acute phase proteins and cell cytochemistry.

Acknowledgements

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Table 6.1. Median values for packed cell volume (PCV), total solids (TS), and white blood cell count (WBC) from inoculated and control RES exposed to FV3-like virus at two different environmental temperatures.

Day	22C			28C		
	Treatment Median	Control	p	Treatment Median	Control	p
PCV (%)						
Pre-exposure	21.13	22.63		18.50	17.25	
Day 4	25.75	19.25		23.75	16.00	
Day 8	13.25	25.00	.02	23.50	19.50	
Day 11	27.50	28.00		17.00	11.00	
Day 15	26.00	26.75		12.75	15.00	
Day 18	30.50	24.00		14.50	24.00	
Day 22	30.50	27.00		16.75	11.50	
Day 25	31.25	16.75		16.00	15.00	
Day 29	ND	ND		26.50	9.50	
TS (mg/dl)						
Pre-exposure	3.78	3.71		2.05	2.40	
Day 4	4.55	3.75		3.35	1.50	
Day 8	3.30	3.80		3.48	1.38	
Day 11	4.55	3.90		3.80	2.60	
Day 15	2.30	4.13		2.35	2.90	
Day 18	2.80	3.70		3.80	3.70	
Day 22	4.40	3.80		1.73	1.30	
Day 25	2.80	3.80		2.58	1.03	
Day 29				3.15	.88	

Table 6.1, continued

	WBC (/μl)					
Pre-exposure	4839.80	5288.19		4109.30	2879.31	
Day 4	8989.43	4554.48		4394.94	1920.24	
Day 8	2808.55	6265.64		5601.19	1716.80	.02
Day 11	8691.05	7000.00		15018.67	1002.22	.02
Day 15	8268.99	4696.18		7453.63	1189.19	
Day 18	10625.19	2660.95	.03	12743.86	4964.93	
Day 22	8000.00	5299.56	.04	5574.73	1771.27	
Day 25	5332.63	3126.04		3614.29	1489.01	
Day 29				13118.89	1581.78	

Table 6.2. Complete blood count parameters from pre-inoculation, initial post-inoculation, and terminal blood samples collected from RES experimentally inoculated with FV3-like virus at two different environmental temperatures.

		Mean/median*	95% CI/ 10-90% percentiles*		Min	Max
PCV	Pre-inoculation	21*	9	22.13*	9	22.5
(%)	Post-inoculation	24.7	14.7	34.6	7	35
	Terminal	19.1	10.3	27.8	3	26
TS^	Pre-inoculation	3.8	2.8	4.7	2.3	5
(mg/dl)	Post-inoculation	3.8	2.6	5	2	5
	Terminal	2.1	1.4	2.9	1	3
WBC	Pre-inoculation	4544.5	3364.7	5724.3	2400	5628
(cells/ul)	Post-inoculation	7389.3	1668.6	13110.1	1203	16264
	Terminal	4073*	1232	6283.4*	1232	12623
Heterophils	Pre-inoculation	1388.5	1016.6	1760.5	933.9	1916
(cells/ul)	Post-inoculation	2429.3	813.9	4044.7	346	4091
	Terminal	1537	620.8	2453.3	344.9	2777.2
Lymphocytes	Pre-inoculation	870.3	184.5	1556.1*	223	1799.4
(cells/ul)	Post-inoculation	944.1*	223	1594.6*	0	3764
	Terminal	372*	24.6	1569.6*	24.6	4544.4
Monocytes	Pre-inoculation	654.9	314.2	995.5	272	1118
(cells/ul)	Post-inoculation	910*	204.8	3167.3*	204.8	3600
	Terminal	805.1	457.1	1153.2	344.9	1205

Table 6.2, continued

Eosinophils (cells/ul)	Pre-inoculation	1331.6	457.5	2205.7	432.8	2207
	Post-inoculation	1377.4	138.3	2616.6	481	3600
	Terminal	418.4	40	1225.9	40	2650.9
Basophils (cells/ul)	Pre-inoculation	244.1*	0	500.1*	0	797.2
	Post-inoculation	837.1	338.3	1335.9	135	1309
	Terminal	487.5*	258.7	942*	258.7	1641

^ Significant difference in mean over time, p=0.034

Table 6.3. Descriptive statistics for complete blood count parameters in eastern box turtles from 2007, 2010, and 2011.

		2007	2010[#]	2011[#]
WBC	Median	5800*	13058*	6488
(cells/ul)	10-90 Percentile	1800 – 23000	3913 – 31639	3411 - 11636
	Min/Max	400 - 64400	1552 - 72160	1342 - 25589
PCV	Median		23	27
(%)	10-90 Percentile		13.1 – 32.0	18.0 – 37.4
	Min/Max		4 - 40	8 - 50
TS	Median		4	3
(mg/dl)	10-90 Percentile		2 – 5	2 – 5
	Min/Max		1 - 7	1 - 6

*Significant difference

between 2007 and 2010, $p<0.0001$

[#]Significant difference between 2010 and 2011 for WBC ($p<0.0001$), PCV ($p<0.0001$), and TS ($p=0.041$)

Table 6.4. Descriptive statistics for differential white blood cells in eastern box turtles from 2007, 2010, and 2011.

		2007	2010	2011
Heterophils	Median	6632	1075	1690
(cells/ul)	10-90 Percentile	2587 – 24127	364 – 2481	669 - 3551
	Min/Max	1071 - 55080	145 - 7884	172 - 8064
Lymphocytes	Median	242	7271	654
(cells/ul)	10-90 Percentile	0 – 1128	1816 – 20527	211 - 1909
	Min/Max	0 - 3240	791 - 46904	0 - 6271
Monocytes	Median	578	285	135
(cells/ul)	10-90 Percentile	97 – 3274	0 – 1248	0 - 609
	Min/Max	0 - 12920	0 - 10490	0 - 2015

Table 6.4, continued.

Eosinophils	Median	1222	1801	1938
(cells/ul)	10-90 Percentile	0 – 1312	497 – 4071	736 - 3953
	Min/Max	0 - 3240	140 - 11205	95 - 14586
Basophils	Median	490	1751	1383
(cells/ul)	10-90 Percentile	0 – 3274	357 – 5367	421 - 3843
	Min/Max	0 - 5328	0 - 21648	24 - 13099

[#]Significant difference between 2010 and 2011 for heterophils ($p<0.0001$), lymphocytes ($p<0.0001$), monocytes ($p<0.0001$), and basophils ($p=0.026$)

Table 6.5. Prevalence of eastern box turtles with intracytoplasmic inclusions in white blood cells sampled in 2007.

Institution	n	Prevalence (%)	95% CI (%)
UT	25	44.0	26.7 - 62.9
WCV	23	4.6	0.8 - 21.8
NCSU	36	5.6	1.5 - 18.2

Figure 6.1. White blood cells from healthy red-eared sliders. Heterophils (A; D, arrowhead), lymphocyte (B, thin arrow), thrombocyte (B, arrowhead), eosinophil (C; D, inverse arrowhead), monocyte (D, three thin arrows), and basophil (E).

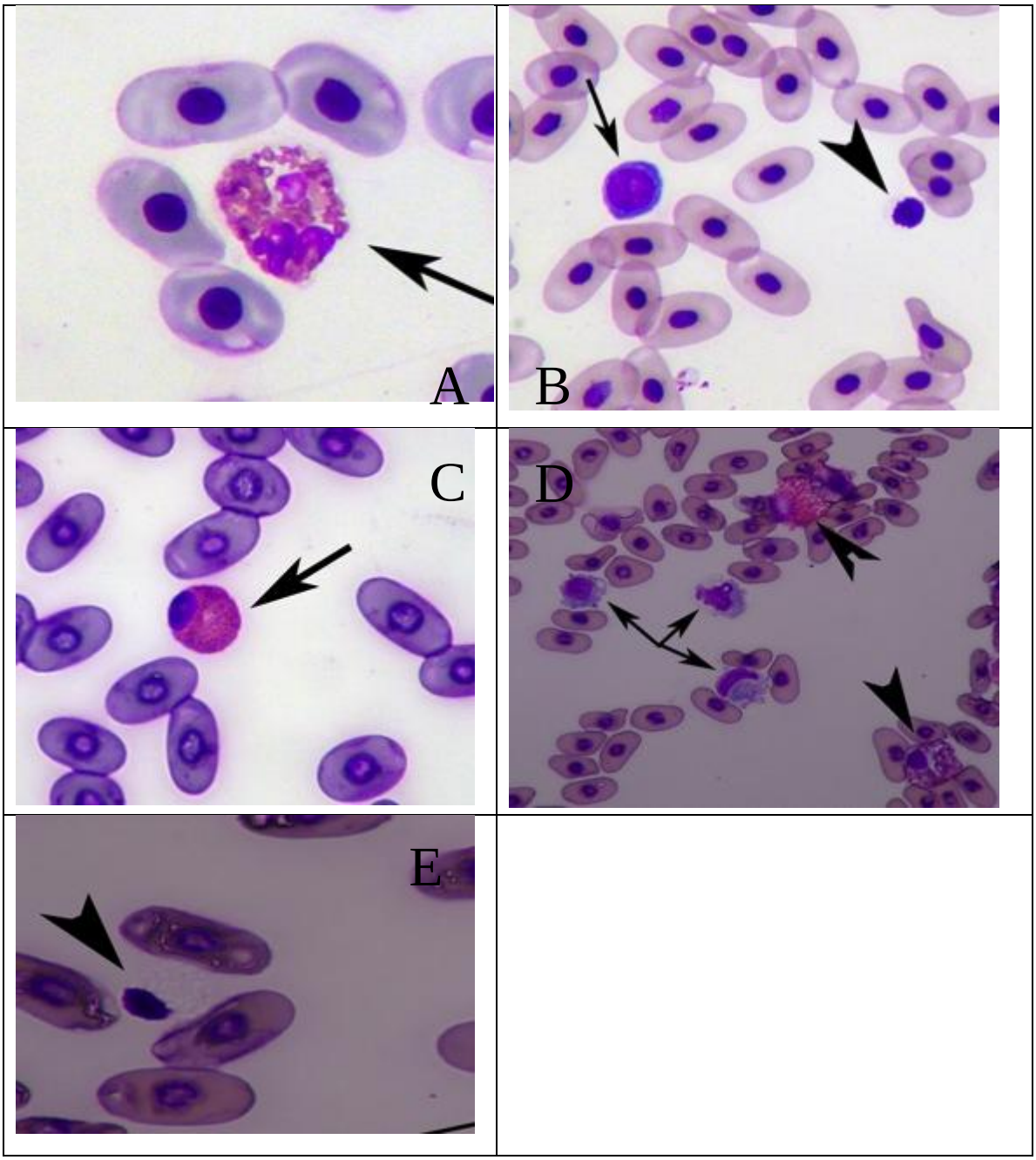


Figure 6.2. White blood cells in apparently healthy free-ranging eastern box turtles. Heterophils (A, thin arrow; C), lymphocyte (A, arrowhead), thrombocyte (B), eosinophil (D), and basophil (E).

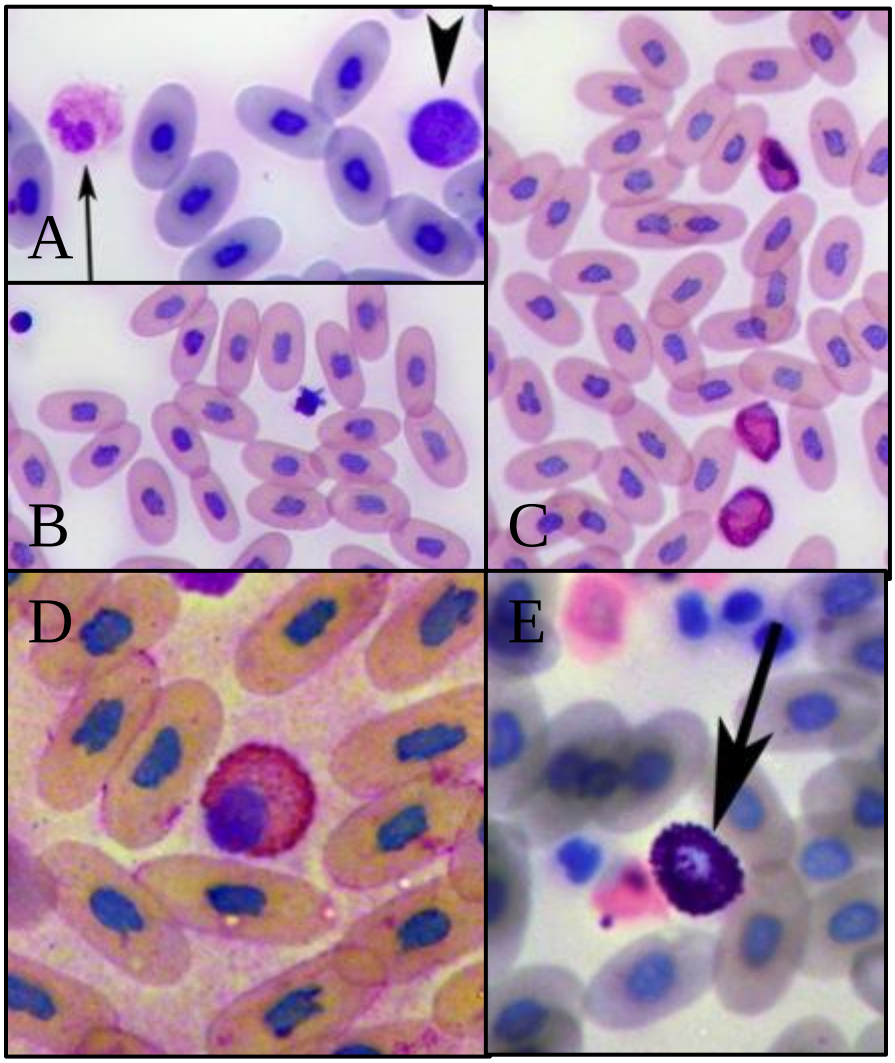


Figure 6.3. Intracytoplasmic inclusions within circulating heterophils (A,B) and monocytes (C,D) observed in red-eared sliders after experimental infection with ranavirus.

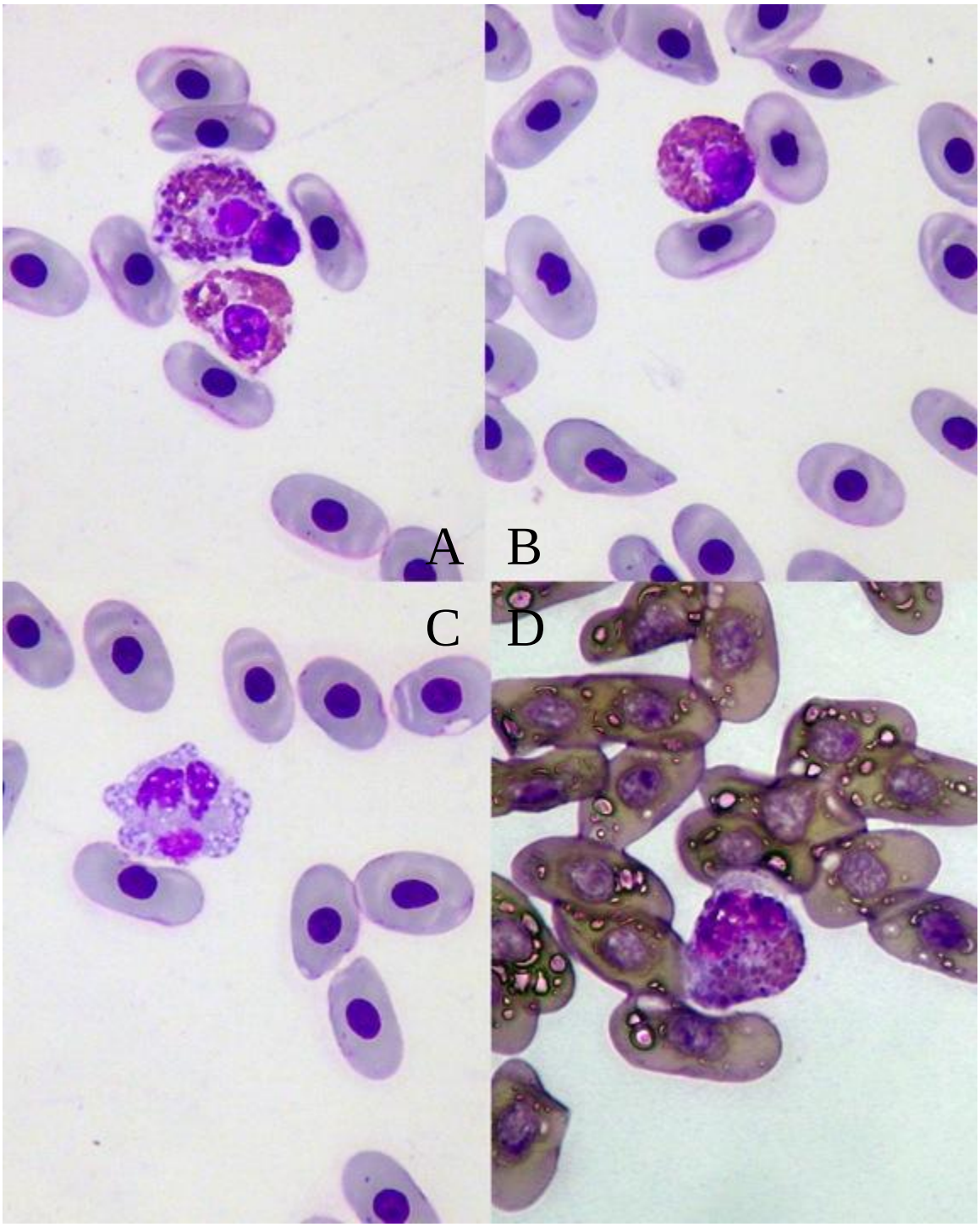
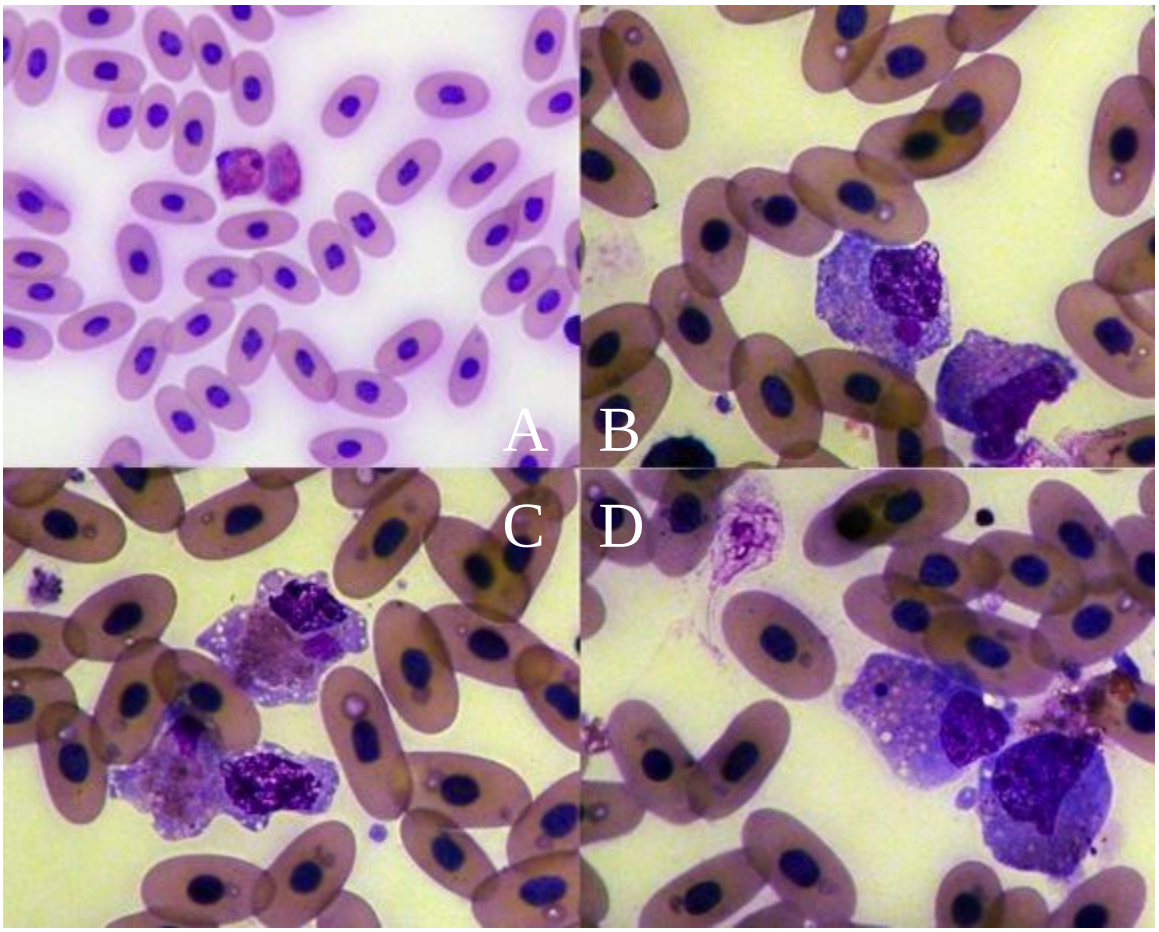


Figure 6.4. Intracytoplasmic inclusions in circulating heterophils (A) and monocytes (B,C,D) observed in eastern box turtles with natural ranaviral infection.



CHAPTER 7

PHARMACOKINETICS OF A SINGLE ORAL DOSE OF ACYCLOVIR AND VALACYCLOVIR IN NORTH AMERICAN BOX TURTLES (*TERRAPENE* SP.)[5]

SHORT COMMUNICATION

Eastern box turtle (*Terrapene carolina carolina*) populations have significantly declined throughout their range (Currylow *et al*., 2011). While a combination of factors are likely playing a role in the declines of the box turtle, disease outbreaks have been emerging across the eastern US in chelonians and may be important (Allender *et al*., 2011; DeVoe *et al*., 2004; Johnson *et al*., 2008).

Herpesviruses and iridoviruses are two common virus families that lead to clinical signs of upper respiratory tract disease in affected chelonians (Allender *et al*., 2011; Brown *et al*., 1999; Harper *et al*., 1982; Johnson *et al*., 2008). Acyclovir has been used anecdotally in the treatment of both viral agents in chelonians (DeVoe *et al*., 2004; Funk & Diethelm, 2006; Marschang et al., 1997); however, to date, only a single pharmacokinetic study has been performed in a single species (Gaio *et al*., 2007).

Acyclovir and its pro-drug valcyclovir are guanine analogue antiviral drugs (Elion, 1993). They are activated by phosphorylation of a virus-specific thymidine kinase (TK). Acyclovir uptake has been shown to be enhanced in herpesvirus-infected cells, with a 10- to 30-fold greater affinity for infected cells than uninfected cells (Beutner, 1995). Once incorporated

[5] Content of this chapter is in press for publication as cited in bibliography as Allender et al., 2012e.

into the viral genome, relatively low levels of the drug are needed to achieve viral inhibition, and adequate intracellular concentrations can be maintained for several hours (Beutner, 1995). Thymidine kinase genes or functional TK enzymes have similarly been identified in iridoviruses (Coupar *et al*., 2005; Jakob *et al*., 2001; Scholz *et al*., 1988; Tsai *et al*., 2005).

The current study evaluated the pharmacokinetics of acyclovir and valcyclovir after a single oral dose in twelve North American box turtles. Animals were housed individually in Vision cages (Model #332, Vision Products Plus, Inc., Canoga Park, CA, USA) that maintained a thermal gradient of 72°F to 87°F. A varied diet of fruits and vegetables was provided every other day. All activities were approved by the University of Illinois IACUC (protocol 11003).

A pilot study using oral acyclovir was initially performed at doses of 40 and 80 mg/kg in one animal each. After the results demonstrated low maximum observed plasma concentration (C_{max}) at the doses used, a decision was made to evaluate the pharmacokinetics of oral valcyclovir at 20 and 40 mg/kg in one animal each. Based on these results, eight animals were administered a single oral dose of 40 mg/kg valcyclovir. Venipuncture was performed via the subcarapacial sinus for all phases of the study. For the final study, blood (up to 0.3 ml) was collected in a 3 ml syringe with a 22 ga needle at 0, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 120 h following oral valcyclovir. Samples were placed in a lithium heparin microtainer (Becton Dickinson, Franklin Lakes, NJ, USA), centrifuged immediately, the plasma placed in a separate cryovial, and stored at -20°C. Samples were transported on dry ice to the Pharmacology Laboratory at the University of Tennessee for analysis.

Plasma samples were analyzed using a reverse phase high-performance liquid chromatography method (HPLC). The system consisted of a 2695 separations module, a 2475 fluorescence detector and a computer equipped with Empower software (Waters, Milford MA,

USA). Valcyclovir and acyclovir were extracted from plasma samples using 1 cc HLB solid phase extraction (SPE) cartridges. The compounds were separated on an Atlantis T3 (4.6 x 100 mm, 5µm) column with a guard column. The mobile phase was a mixture of (A) 10 mM ammonium phosphate pH 2.9 and (B) acetonitrile (97:3). The flow rate was 1.2 ml/min and the column temperature ambient. Fluorescence was measured at an excitation of 260 nm and an emission of 375 nm with a gain of x100.

Standard curves for plasma analysis were prepared by fortifying untreated, pooled turtle plasma with valcyclovir and acyclovir to produce a linear concentration range of 10-1500ng/ml. Average recovery for both drugs was 87% while intra and inter-assay variability were less than 10%. The lower limit of quantification was 10 ng/mL.

Pharmacokinetic parameters for valcyclovir and acyclovir were calculated using WinNonlin 5.2 (Pharsight Corp., Mountain View, CA) (Table 7.1, Figure 7.1). No valcyclovir was detected in any sample and all analysis consisted solely based on plasma acyclovir. Values for plasma half-life (t½), C_{max}, time to maximum plasma concentration (T_{max}), and area under the plasma concentration time curve ($AUC_{0-\infty}$) from time 0 to infinity were calculated from non-compartmental analysis. The AUC and AUMC were calculated using the log-linear trapezoidal rule. Mean residence time (MRT) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. The compartmental pharmacokinetic model was a one-compartment model with first order elimination and weighted 1/Yhat*Yhat and the model was used to simulate the concentration–time profile for several dosage regimens. In this model, it is assumed that K01>>>K10, or that there is no 'flip-flop' effect caused by slow absorption. The best model included an absorption term and biexponential decay. Parameters of this average model were V_F (mL/kg),_K_{01} (1/h), and K_{10} (1/h) which were

29.3971 ± 13.6595, 0.2241± 0.1718, and 0.0456 ± 0.0083, respectively and were used to generate Figure 7.2.

The half-life of acyclovir after a single oral dose of valcyclovir in box turtles was 14.6 h. This is longer than the half-life of acyclovir in humans (3.1 h), cats (3.1 h) and horses (5.05 h) given oral valcyclovir and marginated tortoises (*Testudo marginata*) (8.8 h) given oral acyclovir (Gaio *et al*., 2007; Owens *et al*., 1996; Garre *et al*., 2007; Beutner, 1995). The results demonstrate a slower elimination in box turtles, which could reduce dosing intervals.

Valcyclovir, an esterified version of acyclovir, is rapidly converted to acyclovir after absorption and has greater oral bioavailability than acyclovir (Garre *et al*., 2008). The oral bioavailability of valcyclovir is 3-5 times greater in humans, 8 times greater in horses and 2-3 times greater in cats than oral acyclovir (Garre *et al*., 2007; Nasisse *et al*., 1997). In our pilot study evaluating 80 mg/kg oral acyclovir, our C_{max} was 2200 ng/ml. This was within one standard deviation of our 40 mg/kg valcyclovir dose C_{max}. While bioavailability was not measured in the current study or the previous study in marginated tortoises, this pilot data indicates that, despite a lower dose of valcyclovir, it reached the same concentration as the higher acyclovir dose in box turtles.

Acyclovir has been proposed for treatment of herpesvirus in several species, and for iridovirus in chelonians (Beutner, 1995; Gaio *et al*., 2007). For herpesvirus in humans and horses, inhibitory concentrations need to be maintained above 0.45 μg/ml and 3 μg/ml, respectively (Gaio *et al*., 2007). However, *in vitro* concentrations that completely inhibit tortoise herpesvirus were above 50 μg/ml (Marschang *et al*., 1997). Additionally, *in vitro* studies against an iridovirus indicated only a dose-dependent partial inhibition at 25 μg/ml acyclovir concentration (Johnson, 2006). There have been no *in vivo* studies evaluating efficacy or

inhibitory concentrations in chelonians against either virus. The C_{max} of valcyclovir obtained in this study was 1.94 μg/ml, which is far below the partial *in vitro* inhibitory concentration of the chelonian studies. However, the C_{max} observed in this study is higher than the concentration considered effective for herpes virus infection in humans (0.45 μg/ml), but lower than those shown to be effective in feline herpes virus infection (18 ug/ml) (Beutner, 1995; Owens *et al*., 1996). Future studies should further evaluate *in vivo* efficacy against iridoviruses to determine if higher concentrations of drug are reached in infected cells, as has been demonstrated with herpesviruses in other species.

Toxicity was not specifically evaluated in this study; however, anorexia was seen in two animals, and prolonged lethargy was seen in a turtle administered valcyclovir. This study was performed in October and it is possible that these problems were physiologic responses to the time of year. In cats, nephrotoxicity and bone marrow suppression were seen with repeated dosing of acyclovir and valcyclovir (Owens *et al*., 1996).

In summary, valcyclovir appears to reach therapeutic plasma concentrations in box turtles based on *in vivo* doses effective for herpesvirus infections in humans (0.45 μg/ml), but not *in vitro* studies for herpesvirus or iridovirus in chelonians. However, its increased affinity for infected cells may allow intracellular concentrations to reach therapeutic levels for these pathogens when plasma concentrations do not. A simulated oral dose of 40 mg/kg every 24 h is predicted to maintain concentrations above 0.45 μg/ml in the average animal. Some limitations of our predictions include the assumption of linear pharmacokinetics within the range of simulated concentrations, the unknown effect of inter-individual pharmacokinetic variability in the population of turtles (temperature, season), the lack of bioavailability data, and the application of a human therapeutic window to turtles. Therefore, although our selected dosage

regimen is a reasonable starting point, pharmacodynamic and repeat dosing pharmacokinetic studies are needed to further characterize optimal dosing of acyclovir in turtles.

In conclusion, this study provides valuable insight into the treatment of clinically important diseases in this species. Furthermore, it provides a scientific rationale for development of other dose regimes for related species.

Table 7.1. Pharmacokinetic parameters (mean ± SD) of acyclovir in the plasma of eight box turtles after a single oral dose (40 mg/kg) of valcyclovir.

Pharmacokinetic parameter	Acyclovir
$t_{½}$* (h)	15.2 ± 2.3
T_{max} (h)	13.0 ± 7.0
C_{max} (µg/mL)	1.94 ± 0.81
$AUC_{0-\infty}$ (h·µg/mL)	42.0 ± 15.3
$AUMC_{0-\infty}$ (h·h·µg/mL)	958.9 ± 307.5
$MRT_{0-\infty}$(h)	23.1 ± 3.4
V_F (mL/kg)	29.3971 ± 13.6595
K_{01} (1/h)	0.2241± 0.1718
K_{10} (1/h)	0.0456 ± 0.0083
Cl_F (mL/h/kg)	1116 ± 370
K_{01} $T_{½}$ (h)	3.1 ± 1.0
K_{10} $T_{½}$ (h)	17.2 ± 3.6

* Harmonic mean

Figure 7.1. Mean ± SD acyclovir plasma concentrations (µg/ml) following oral administration of valcyclovir (40mg/kg) to eight box turtles.

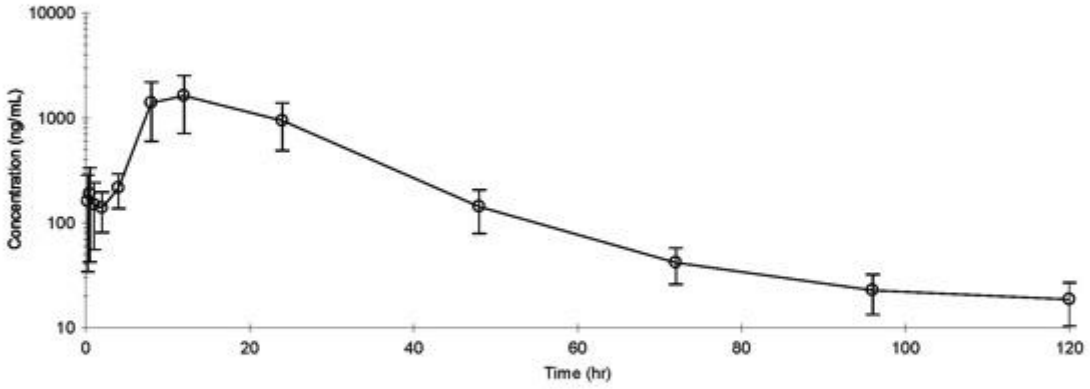
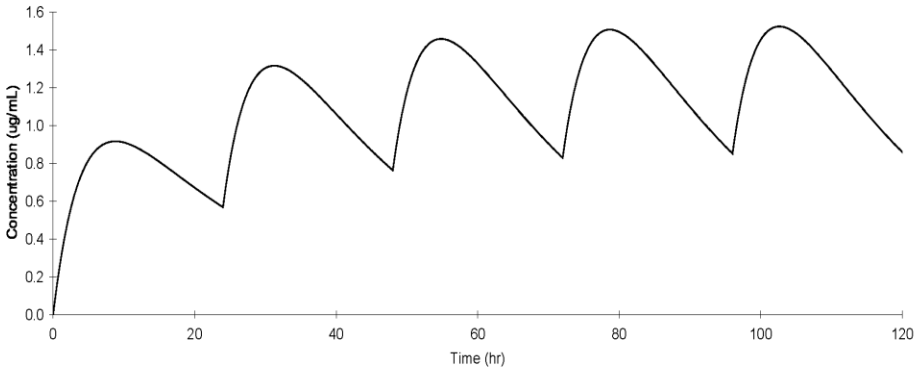


Figure 7.2. Simulated average pharmacokinetic profile of plasma acyclovir concentrations (µg/ml) after oral administration of valcyclovir at 40 mg/ml every 24 hours.



CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

Chelonians are experiencing significant population declines, and the impacts of disease on limiting biodiversity in this Order have been under-investigated . To better determine the effect of disease on biodiversity, the epidemiology of important diseases need to be adequately characterized. The research in this dissertation contributed to the further characterization of the epidemiology of ranaviral disease in chelonians and can be used to develop a better understanding of the diagnosis, transmission, and management of this virus in captive and free-ranging populations.

Three quantitative PCR assays were developed that can detect ranaviral DNA. From this, the TaqMan PCR assay was found to be highly sensitive and specific for use in chelonians and can detect viral DNA in small quantities. However, based on the transmission study, it appears that detection of small quantities of virus may not be required to detect infection. Animals experimentally challenged with ranavirus either had below the detectable limit of copies (<529 in most assays) or had multiple million to billions of copies. The detection limit of conventional PCR is 529,000, which is more than adequate to determine clinical infection. Quantitative PCR, however, would be useful in determining lower viral quantities in tissues that may be involved in viral persistence or replication. It was determined that viral copy number was highest in the kidney in this study, which is similar to the findings in previous studies with *Xenopus*. In this anuran, the kidney was identified as a tissue for viral persistence. Evaluating whether this is true in chelonians is needed, and the application of this qPCR assay for serial detection of viral quantities in the kidneys and other tissue samples may allow for that determination in chelonians.

Additionally, this qPCR might help to identify subclinical carriers or reservoirs that haven't been identified yet. To date, no chelonian species has had detectable levels of ranavirus on PCR without evidence of disease, but future surveillance studies should investigate multiple species and then follow up in those species that have presence without disease with appropriate transmission studies.

While this dissertation provides new insight into the epidemiology of ranaviral disease in chelonians, there is much more that needs to be investigated. To date, there have been numerous reports of ranavirus outbreaks in amphibians; however, no such outbreaks have been documented in free-ranging chelonians. The reasons for this are potentially varied, but may be attributed to the fact that chelonians are: 1) a spill-over host and are not affected either in great numbers or it is difficult to identify affected animals, 2) chelonians are a reservoir host that fail to develop clinical signs unless immunocompromised, or 3) the mechanisms of transmission are different in chelonians and this provides some protection to disease. It is my opinion that outbreaks may very well occur, but go unnoticed because of the natural history characteristics (e.g., solitary lifestyle, deep aquatic habitat, wide home ranges) of these animals as well as the absence of many long-term studies evaluating the health of chelonians. This study described a component of a long-term health project monitoring box turtles in Tennessee that began in 2008 and has recently been funded through 2015. The study has also sampled aquatic terrapins in surrounding water bodies, in which a positive red-eared slider turtle was detected. Expanding this study to include additional species as well as continuing to monitor box turtle health will be helpful in further characterizing the epidemiology of ranavirus in chelonians.

One aspect of the epidemiology of ranavirus that deserves further investigation is the possibility that invasive chelonian species are responsible for the introduction and persistence of

the virus in the environment. It has been shown that translocation of tiger salamanders from the Midwest to Arizona has caused nearly annual ranaviral outbreaks in native amphibians. A reptile species that is similarly invasive and expanding in range is the red-eared slider turtle. This turtle species has been implicated for introducing parasites in previously naïve native turtles, so it is feasible it could similarly introduce a virus (Verneau et al., 2007). The results of the experimental infection study in this dissertation confirm that red-eared sliders are susceptible to ranavirus infection, but provided additional insight into the effect of environmental temperature on the pathogenesis of the virus. Reptiles are ectotherms and are physiologically and immunologically dependent on their environment. Viruses, likewise, are heavily influenced by the environment(s) in which they are found. Ranaviruses are known to thrive in cooler temperatures, and the results of this study further support this as it relates to a chelonian host, with more severe disease and mortalities in animals held at cooler temperatures. The effect of temperature may also play a role in the persistence of the virus and transmission of the virus among chelonians in the environment. In this dissertation, a constant temperature was maintained throughout the entire experimental period. However, in a free-ranging setting, temperature fluctuates, sometimes dramatically, throughout the day. This may lead to differences in both viral replication and immune response in the reptilian host that may protract the disease and allow for persistent shedding into the environment. Additional transmission studies should be performed to further elucidate the role of temperature on the interactions between Ranavirus and chelonians.

The development and refinement of additional diagnostic assays would be useful in further characterizing the response of chelonians to ranavirus infection/exposure. This should combine both pathogen- and pathogen-exposure assays. Pathogen-based assays such as qPCR,

virus isolation, IHC, EM, and histopathology are useful in not only determining whether ranavirus is present, but if it is active. Many of these assays have an ideal sample type or assay conditions that need to be validated in chelonians. Future studies that develop an IHC for ranavirus in turtles would be useful in examining retrospective samples that might frame the historical relationship of ranavirus and chelonians. A pathogen-exposure assay was attempted in this dissertation, but was unsuccessful due to the lack of a positive control. A serologic assay would be valuable in screening populations where reservoirs animals exist, if indeed they do. However, based on our current understanding of this virus in chelonians, the virus appears to cause acute disease and high mortalities. Therefore, a serologic test would likely have limited value.

Additionally, it appears that ranaviral disease in reptiles is an acute disease in chelonians and many animals may fail to develop a humoral response before they die. It is possible that red-eared sliders require more than 30 days to mount an antibody response, as it has similarly been determined that tortoises take 6 to 8 weeks to mount an immune response to *Mycoplasma* infection. Alternatively, it is possible that a cell-mediated response is responsible for the protection to ranavirus in chelonians, which has been described as a component of the immune response in *Xenopus*. However, *Xenopus* also produces an antibody response, but it is not detected until after a second infection. Therefore, future experiments should challenge turtles with a non-lethal ranavirus infection and then re-infect after a period of time and investigate for presence of antibodies.

Other non-specific mechanisms that characterize the immune response to ranavirus in turtles are also needed. Hematology was shown to not be helpful in diagnosing infections, which has been my experience with other reptilian diseases as well. However, it is possible that a spike

in WBC or other changes might have been missed by the twice-weekly sampling intervals. Future studies should sample more frequently after infection to determine if a measurable response is observed. One aspect of the clinical pathology that did show changes was the measured total solids. Total solids are composed of many different types of compounds, mainly proteins, and including immunoglobulins. Future studies should investigate not only protein electrophoresis to characterize the immunologic response, but also acute phase proteins. Acute phase proteins are well-conserved among all vertebrates, and have been used to characterize acute disease responses in these animals. Currently, there are plans to add this diagnostic test to the ongoing long-term box turtle study in Tennessee.

The treatment of ranavirus in chelonians has not been successful for most cases. The development of therapies will be useful in managing captive cases, but also may be useful in managing endangered populations of free-ranging animals. Valcyclovir was shown to be detectable in box turtles, but its therapeutic use needs to be evaluated in a pharmacodynamics study. Additional treatment modalities need to be investigated as well, including the modulation of temperature. In a study that accidentally induced natural ranaviral disease when red-eared sliders were exposed to 16°C, the course of clinical signs reversed and resulted in only one mortality after the environmental temperature was changed to 28°C. This preliminary study indicates that temperature manipulation shows promise as a reasonable approach to managing captive animals, and that natural fluctuations in environmental temperature may serve as a control method for this virus in wild chelonians. Future studies should perform similar treatment studies at a range of environmental temperatures to assess their effectiveness. Additionally, spatial and temporal studies characterizing the effects of ranavirus in wild populations of chelonians should evaluate the influence of temperature on outcome.

In conclusion, the research in this dissertation answered several questions about the epidemiology of ranavirus in chelonians. But like any good project, it created more questions that need to be answered. Future research should investigate this disease and others that might be present and yet undetected. Surveillance for emerging pathogens is critical for the conservation of any species. The research presented here can be used as part of population management and planning, but also as a template to investigate the health and diseases of other reptile populations.

CHAPTER 9

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APPENDIX A

METHODS TO DEVELOP AN ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF ANTIBODIES TO RANAVIRUS IN EASTERN BOX TURTLES AND RED-EARED SLIDERS

Introduction

The benefit of measuring exposure to infectious diseases cannot be understated. Enzyme linked immunoassays (ELISA) have been previously used to successfully detect antibodies to different pathogens in chelonians (Ariel et al., 1995; Johnson et al., 2006). While ELISA has been used in one study to detect *Ranavirus* exposure in gopher tortoises and one population of repatriated box turtles in Pennsylvania (Johnson et al., 2010), no wide scale effort has been performed in free-ranging box turtles in the southeast US. Utilization of this assay is extremely important for diseases, such as *Ranavirus*, that have high mortality in naive individuals, but potentially low morbidity during subclinical infections or reservoir individuals. It is crucial to identify exposure in these individuals to determine a more accurate prevalence of the disease and potential risk to the population.

Our specific hypotheses: A newly developed enzyme-linked immunosorbent assay (ELISA) will be at least 80% sensitive and specific for detection of anti-*Ranavirus* antibodies, respectively.

Materials and Methods

Viral Isolate

A *Ranavirus* isolated and partially characterized from a naturally infected box turtle in Tennessee was used as the antigen in the development of the ELISA. Briefly, polymerase chain reaction (PCR) targeting a portion of ranaviral major capsid protein genes followed by nucleotide sequencing demonstrated that the isolate shared 100% sequence identity of approximately 500 bp with the major capsid protein gene of Frog Virus 3.

Antigen Preparation

The *Ranavirus* isolate was grown in *Terrapene* heart cells (TH-1). Flasks were scraped when cells exhibited 100% cytopathic effects. Uninfected flasks were concurrently processed in the same manner to serve as control antigen to detect any background cross reactivity of plasma to cellular proteins. Cells and media were then transferred to 15ml tubes, centrifuged at 4,500x*g* for 30 minutes, and the supernatant discarded. The cell pellets were resuspended in residual media, frozen and thawed three times, thoroughly vortexed before and after each freeze cycle, and centrifuged again at 4,500x*g* for 30 minutes. PCR was performed to confirm the presence of viral DNA.

ELISA Methods and Results Timeline

An indirect ELISA was attempted to determine the presence of anti-*Ranavirus* antibodies similar to previous reports in red-eared sliders and ornate box turtles (Johnson et al., 2010) and similarly used in identifying the presence of anti-tortoise herpesvirus and Mycoplasma antibodies in tortoises (Origgi et al., 2004). The FV3 isolate was used as the antigen in the assay.

Mar 2011. *Checkerboard*. Initially, each well of a 96 well high-protein binding microplate (Immulon HB4X) was coated overnight at 4°C with 50 µl of two-fold serial dilutions 1:50 through 1:800 dilution of either an uninfected lysate from *Terrapene* heart cells (TH-1, ATCC-CCL 50, American Type Culture Collection, Rockville, MD) or TH-1 cell lysate from cells

infected with FV3. Lysates were diluted in 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 NaCl and 0.02% NaN_3 (PBS/Azide). Wells were washed four times in ELISA wash buffer (PBS/Azide with 0.05% Tween-20). This washing process was repeated in between all of the following steps. Wells were blocked with 300 µl of Superblock blocking buffer (Pierce) for one hour at room temperature. All remaining steps will be incubated for one hour at room temperature. Positive plasma sample ("Old Lady" box turtle – from University of Florida) were added in 50µl volumes at a two-fold serial dilutions from 1:50 to 1:1600 in blocking buffer. The secondary antibody used was a biotin-conjugated mouse anti-tortoise immunoglobulin (Ig) monoclonal antibody diluted to a final intended concentration of 1µg/ml in blocking buffer, however initial dilution was inadvertently calculated to 0.1 µg/ml. Alkaline phosphatase-conjugated-streptavidin (Zymed Laboratories, Inc., San Francisco, CA) was applied to each well at 50 µL of a 1:2500 dilution in PBS/Azide. Next, 200 µl of a 1.0 mg/ml P-nitrophenyl phosphate (Ready to use formula, MP Biomedicals). The optical density (OD) of each well will be read at A405 using a BioTek Synergy 2 ELISA reader microplate reader (Awareness Technology, Palm City, Florida, USA) after 30 minutes. ***– No absorbance above baseline was detected in any sample.***

March 2011. Enzyme reaction test. Tested enzyme reaction step of alkaline-phosphatase streptavidin with pNPP in eppendorf tube. Added 5 ul AP-streptavidin with 150 ul and 200 ul of pNPP in separate tubes, respectively. ***Both turned a bright yellow color***. Then diluted the AP-streptavidin 1:1000 and 1:2500 and added to 200 ul of pNPP. ***Both turned yellow after 20-30 minutes***.

March 2011. Checkerboard. Created another checkerboard of positive and negative cell lysates (dilutions of 1:50 through 1:800) with positive ("Old Lady") and negative turtle plasma (dilutions 1:50 through 1:800). **– *No absorbance above baseline was detected in any sample.***

April 2011. Troubleshooting plate. Loaded plate with serial dilutions of FV3 and turtle plasma directly (two-fold serial dilutions 1:10 through 1:320). Turtle plasma was a mixture of plasma from 15 turtles diluted in superblock buffer. Used 3 dilutions of monoclonal: 0.5 ug/ml, 1 ug/ml, 2 ug/ml. Diluted AP-streptavidin 1:1000 in PBS/Azide. **– *No absorbance above baseline was detected in any sample.***

April 2011. Checkerboard. Determined that original dilution of monoclonal was off by a factor of 10. Diluted monoclonal to 1 ug/ml and repeated checkerboard with plasma mixture and FV3. **– *No absorbance above baseline was detected in any sample.***

June 2011. Troubleshooting plate. Made fresh dilutions of turtle plasma in PBS/Azide in serial two-fold dilutions 1:8 through 1:4096. Made fresh dilutions of monoclonal antibody 1 ug/ml to 2 ug/ml. Made fresh dilution of AP-streptavidin 1:1000 in PBS/Azide. Loaded rows A and B directly with turtle plasma mixture, row C directly with 1 ug/ml monoclonal, row D directly with 2 ug/ml monoclonal, rows E and F directly with AP-streptavidin 1:1000. ***All wells showed a color change. This indicates that 1) the interaction between the unknown turtle plasma and monoclonal worked; 2) the monoclonal reacted with the streptavidin and; 3) the enzyme reaction between the streptavidin and pNPP worked.***

June 2011. Checkerboard with FV3 antigen dilutions and positive turtle plasma ("Old Lady"; "Smudge sample" – University of Florida; RES (+) – University of Florida). ***– No absorbance above baseline was detected in any sample****.*

July 2011. Checkerboard and troubleshooting. Made fresh dilutions of all buffers. Made fresh dilutions of antigen, positive and negative controls, monoclonal antibody, and AP-streptavidin. Obtained new ELISA plate (Nunc Maxisorp) that was used in previous published report (Johnson et al., 2010). Loaded row A through D with FV3, row E with turtle plasma, row F with monoclonal, and row G with streptavidin. ***– No absorbance above baseline was detected in any sample loaded with FV3. All troubleshooting wells loaded directly with turtle plasma, monoclonal, and AP-streptavidin was observed WITH a color change. This confirmed that the antigen or antigen-positive antibody complex were at fault. Need to investigate other potential positive controls as the ones used may not be good again.***

December 2011. New potential positive controls and new FV antigen. Received plasma from 14 captive eastern box turtles form the Maryland Zoo that were collected in October 2011 (Appendix B). The collection of turtles had experienced a ranavirus outbreak during June through August 2011. Prepared dilutions of two formulations of FV3 (scraped cells frozen and thawed, in addition to previous method of freeze/thaw cell culture flask). Loaded rows A through C with FV3 antigen dilutions and Maryland zoo plasma dilutions. Loaded rows D and E with Maryland zoo turtle plasma, row F with monoclonal, and row G with AP-streptavidin. ***– No absorbance above baseline was detected in any sample from rows A through C. Rows D through G all showed a color change. This demonstrated that the ELISA worked for the same turtle plasma that failed to react with FV3.***

January 2012. New positive controls. Tested plasma from experimentally infected red-eared sliders. Plasma was harvested from all six turtles that became infected from the last time they were sampled. ***– No absorbance above baseline was detected in any sample, except for troubleshooting rows (see above).***

Conclusions and Future Directions

The ELISA that attempted to detect anti-FV3-like virus antibodies in turtles in this study failed to work. The troubleshooting identified either the viral antigen or the positive control as the steps that failed. FV3-like virus has been shown to be in the enveloped or non-enveloped form. It is possible that virus isolation produced either form exclusively and that antibodies are only made to the opposite form. However, virus isolation techniques were similar to other reports. Alternatively, it is possible that the FV3 antigen failed to bind to the plate. However, this appears unlikely because other proteins (unknown plasma, monoclonal) on the same plate bound well and were detected.

Even though the FV3-like viral antigen may be the cause of failure, the more probable cause is the lack of a true positive control. As has been described in this dissertation, the acute nature of the disease may fail to allow enough time to produce antibodies. And the original positive controls that were used in this study were generated from other studies at the University of Florida and were several years old and stored in blocking buffer. This may have rendered these positive controls ineffective. Additionally, it has been shown that primary infection of FV3-like virus does not produce antibodies in *Xenopus*, but upon reinfection an active IgY response occurs. This could account for why our experimentally infected turtles failed to react.

Future studies should evaluate several possibilities: 1) Determine using electron microscopy if the FV3-like virus is bound to the ELISA plate; 2) Determine the form (enveloped or non-enveloped) of virus that is present in the sample; and 3) Develop a true positive control by following experimentally infected turtles out to 3-4 months and during re-infection.

APPENDIX B

BRIEF DESCRIPTION OF THE APPLICATION OF A NEWLY DEVELOPED QPCR FOR MONITORING AN ONGOING OUTBREAK OF FV3-LIKE DISEASE IN A CAPTIVE EASTERN BOX TURTLE COLLECTION

Introduction

Twenty-two eastern box turtles from the Maryland zoo began to show signs of ranaviral infection during the summer of 2012. Initial diagnosis was made on necropsy tissues using conventional PCR at the University of Florida. After confirmation, qPCR that was developed during this dissertation (Allender et al., 2012a) was applied to monitor the progression of disease. This is a brief report that describes the methods and results that were performed.

Materials and Methods

Quantitative PCR. DNA was extracted from swabs and whole blood using the DNA mini kit following the manufacturer protocols (Qiagen, Valencia, CA, USA). Real-time qPCR assays were performed using a real-time PCR thermocycler (7500 ABI, MFR) and data was analyzed using associated software (SDS, MFR). Sense (GCTGCCAAGATGTCGGGTAA) and anti-sense (AACGCCGACCGAAAACTG) primers were used to identify a 65 bp segment of the frog virus 3 major capsid protein. Each TaqMan reaction contained 12.5 µl TaqMan Platinum PCR Supermix-UDG with ROX, 1.25 µl TaqMan primer-probe, 2.5 µl FV3 dilution, and water to a final concentration of 25 µl. Cycling parameters for all assays (SYBR green and TaqMan) were as follows: 1 cycle at 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles at

95°C for 15 seconds and 60°C for 60 seconds, and a final cycle of 72°C for 10 minutes. A standard curve was performed on each 96 well plate using a control plasmid containing FV3.

Statistical Analysis. Categorical variables were evaluated over time using the Cochran's Q test. Specific between group differences were evaluated using the McNemar test. Non-normally distributed continuous variables were evaluated over time using the Freidman's ANOVA. Specific between group differences were evaluated using the Wilcoxon Signed Ranks test.

Results

There were significant differences over time in prevalence ($p<0.0001$). The prevalence of ranavirus was higher in July than late August ($p=0.39$) and September ($p=0.001$), and between early August and September ($p=.031$). There was no difference between the prevalence of ranavirus in July and early August ($p=0.219$), early August and late August ($p=0.25$), and late August and September ($p=0.25$). (Table 1).

The number of viral copies were significantly different over time ($p<0.0001$) (Table 2). There were significantly more ranaviral copies detected in July than September ($p=0.003$) and early August than September ($p=0.028$). There were no other significant differences between dates in viral copies (Table 2). Of positive animals, median viral copies were highest in late August (Table 3).

Table B.1. Prevalence of ranavirus DNA detected from oral swabs using quantitative PCR in captive eastern box turtles.

Date	n	Positive (%)	Negative (%)
July	22	72.7	27.3
August (early)	18	50	50
August (late)	16	31.3	68.8
September	15	0	100

Table B.2. Number of ranaviral copies detected from oral swabs determined by quantitative PCR in all positive and negative captive eastern box turtles.

Date	n	Median	10-90% iles	Min-Max
July	22	78558.22	0 – 2.16 * 10^9	0 – 5.12 * 10^9
August (early)	16	66937660.5	0 – 1.44 * 10^9	0 – 4.78 *10^9
August (late)	15	925348.3	0 – 7.78 * 10^8	0 – 2.096 *10^9
September	15	0	0 - 0	0 - 0

Table B.3. Number of ranaviral copies detected from oral swabs determined by quantitative PCR in only positive captive eastern box turtles.

Date	n	Median	10-90% iles	Min-Max
July	16	7062831.6	$14271.77 – 3.36 * 10^9$	$8097.25 – 5.12 * 10^9$
August (early)	9	91085900	$1850696.6 – 4.78 * 10^8$	$1850696.6 – 2.096 * 10^9$
August (late)	5	2463556.8	$78753.23 – 2.39 * 10^9$	$78753.23 – 4.79 * 10^9$

APPENDIX C

DEVELOPMENT AND VALIDATION OF TWO SYBR GREEN QUANTITATIVE PCR ASSAYS FOR DETECTION OF FROG VIRUS 3-LIKE VIRUS IN EASTERN BOX TURTLES (*TERRAPENE CAROLINA CAROLINA*)

Abstract: *Ranavirus* has caused disease epidemics and mass mortality events globally in free-ranging fish, amphibian, and reptile populations. Viral isolation and conventional PCR are the most common methods for diagnosis. In this study, quantitative real-time PCR (qPCR) assays were developed using two distinct SYBR Green primer set assays all derived from a highly conserved region of the major capsid protein of frog virus 3 (Family *Iridoviridae*, genera *Ranavirus*). Standard curves were generated using each primer set from a viral DNA segment cloned within a plasmid. One SYBR Green assay detected viral DNA 1000 times lower than conventional PCR. Thirty-one clinical samples (whole blood and oral swabs) from box turtles were tested using these assays and the prevalence of the virus determined. Quantitative PCR allows for a superior, rapid, sensitive, and quantitative method for detecting *Ranavirus* in box turtles, and this assay will be useful for early detection and disease monitoring.

Introduction

Diagnostic assays that are validated and optimized to detect the presence of pathogens in certain species are key to characterizing the disease and understanding disease ecology. Quantitative real-time PCR (qPCR) has been previously developed for detection of ranavirus in turtles using TaqMan (Allender et al., 2012a).

The purpose of this study was to develop and evaluate different diagnostic methods for characterizing an emerging pathogen, frog virus 3-like virus (FV3; genera *Ranavirus*, Family *Iridoviridae*) in box turtles. The hypotheses tested in this study were that qPCR SYBR green based assays would be both sensitive and specific for characterizing FV3-like virus in box turtles. This is essential when considering the application of these assays to additional free-ranging populations and/or experimental models. Furthermore, it allows for evaluating potential climatic and environmental impacts of the disease, treatment, and management options.

Materials and Methods

DNA extraction, conventional PCR, sequencing, and cloning was performed as previously described for these same samples (Allender et al., 2012a).

SYBR qPCR Assay

Primer sets for SYBR green (SYBR-BTR 1 and 2) based qPCR were designed using a commercial software program based qPCR assay were designed using a commercial software program (Primer Express®, Applied Biosystems, Carlsbad, CA) based on published sequences of the major capsid protein of FV3 (Mao et al., 1997). Real-time qPCR assays were performed using a real-time PCR thermocycler (7500 ABI real-time PCR System, Applied Biosystems, Carlsbad, CA) and data was analyzed using associated software (Sequence Detection Software

v2.05, Applied Biosystems, Carlsbad, CA). Each SYBR-Green reaction were performed with a 25 µl total reaction containing 12.5 µl of 2X SYBR green Mastermix,[i] 0.25 pmoles sense and anti-sense primers, 2.5 µl turtle-derived FV3 dilution, and 9.5 µl dH_20. Cycling parameters for all assays were as follows: 1 cycle at 50°C for 2 minutes followed by 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds, and a final cycle of 72°C for 10 minutes.

Standard curve, Specificity, and Sensitivity

Specificity of positive results from SYBR green reactions were confirmed on purified and cloned PCR products using sequencing. To determine the sensitivity, assays were performed in three technical repeats on dilutions of turtle-derived positive control plasmid of FV3 MCP DNA ($5.29x10^9$ - $5.29x10^1$ copies/rxn) within a single run. Standard curves were generated using the cycle threshold (Ct) values of the positive control plasmid dilutions. Intra-assay variation was determined for both assays by calculating the mean Ct values, standard deviations (SD), and coefficient of variations (CV) separately for each control plasmid DNA dilution.

Box turtle samples

Thirty-one eastern box turtles that were presented to the University of Tennessee College of Veterinary Medicine Wildlife clinic were examined during March through October 2007. Whole blood and swabs of the oral cavity were collected as previously described (Allender et al., 2011). The University of Tennessee Institutional Animal Use and Care Committee approved all animal use (protocol 1864). Whole blood and oral swab extracts of samples from animals with unknown disease status were evaluated using both SYBR-BTR 1. Results for detection were compared to published conventional PCR results for the same samples (Allender et al., 2011).

Statistical Analysis

The quantity of ranavirus target DNA in infected whole blood and oral swabs was determined using a standard curve method. The copy number of the target DNA was determined from the standard curve generated with ten-fold dilutions of the positive control plasmid that contained the target sequence of the respective qPCR assay.

Copy numbers were tabulated and evaluated for normality using the Shapiro-Wilk test. Mean, median, standard deviation, 95% confidence interval, and 10-90% percentiles were determined for positive cases (copy number) for each assay. The Mann-Whitney U test was used to evaluate between assay differences. The prevalence of ranavirus was determined for each assay (categorical variable assigned; 1=positive, 0=negative). Exact 95% binomial confidence intervals were determined for all proportions. Level of agreement (kappa) was determined between both the real-time PCR assays and the conventional PCR previously reported based on prevalence. All statistical analysis was performed using statistical software (IBM SPSS Statistics 20, Chicago, IL).

Results

Conventional PCR was performed to evaluate turtle-derived FV3-like virus dilutions using both MCP 4 and SYBR- BTR 1. The SYBR primer sets were designed to detect various length gene segments of a conserved portion of the MCP gene of FV3 (Table 1). The SYBR-BTR 1 primer pairs successfully amplified the 113 bp segment with a single melting point of 84.90 ± 0.26°C (Figure 1a). The SYBR-BTR 2 primer pairs amplified a 110 bp segment with a single melting point of 86.28 ± 0.37°C (Figure 1b). These results were validated as specific amplifications with sequencing and gel electrophoresis (Figure 4).

Serial ten-fold dilutions of positive control plasmids were assayed with SYBR- BTR 1 and SYBR- BTR 2 primer pairs and standard curves were generated based on Ct values (Figure 2). The linear range for SYBR-BTR 1 and SYBR-BTR 2 qPCR was seen between 5.29×10^9 to 5.29×10^4 viral copies with coefficients of variation (R^2) of 0.997 (slope = -3.164) and 0.997 (slope = -3.182), respectively.

The intra- and inter-assay reproducibility was evaluated for the serial dilutions of the control plasmids (Table 2). The intra-assay CVs for SYBR-BTR and SYBR-BTR 2 were between 0.06 – 0.57% and 0.07 – 0.59%, respectively. The inter-assay CVs were 0.1 – 0.68% and 0.19 – 3.66%, respectively. These results indicate high reproducibility between assays for SYBR-BTR 1 at all dilutions and for dilutions with greater than 10^4 concentration of FV3 in SYBR-BTR 2.

When using conventional PCR to amplify the gene segments and gel electrophoresis, the level of detection using MCP 4 and SYBR-BTR 1 primers was 5.29×10^5 and 5.29×10^3 viral copies per reaction, respectively. The dynamic range for qPCR assays at which point the Ct value of the triplicates were variable or amplification plot was reliable was from 5.29×10^9 to 5.29×10^1 for SYBR-BTR 1 and 5.29×10^9 to 5.29×10^4 for SYBR-BTR 2.

Box turtle samples

Twenty-nine blood samples and thirty oral swabs were collected routinely from turtles presented to the University of Tennessee from March through October 2007. Characteristics from these samples are presented elsewhere (Allender et al., 2012a) Quantitative PCR determined the presence of ranavirus in blood was 3% (95% CI:0-9) using the SYBR-BTR primer set. Prevalence of ranavirus in the oral swab samples was also 3% (95% CI:0-9). Mean

viral quantity in blood for the single animal that was positive using both SYBR-BTR was 2.67 x 10^8. The mean viral copies in the swab sample from the same individual were 2.8 x 10^9.

Discussion

Within each assay there was a difference in level of detection (dynamic range) as well as specificity. The level of detection determined by C_t value, low CV, and melting curve analysis were 5.29 x 10^5 and 52 for the SYBR-BTR 2 and SYBR-BTR, respectively. The level of detection included a lower viral copy than the linear range for each of the three assays. The linear range was more reliable for quantification of higher viral copies than lower viral copies. It is likely that primer concentrations need to be optimized for low viral copy numbers, resulting in a separate protocol based on viral copy. Future studies should lower primer concentrations in assays with viral copies less than 5.29 x 10^4 to establish a linear range that is more reliable for quantification. In the absence of that optimization, the developed assays are reliable for absolute quantification of ranaviral DNA, with greater than 5.29 x 10^4 viral copies and relative quantification for the entire dynamic range.

Critical threshold values were lower in both SYBR probes than the TaqMan assay previously reported (Allender et al., 2012a). Non-specific products, such as primer-dimers, are quantified in SYBR assays and are likely responsible. Additionally, TaqMan assays utilize a third selection step (probe), which provides for greater specificity not seen with SYBR assays. The higher C_t values seen at the same dilutions may be due to either amplification being slowed in this TaqMan step or due to the lack of strong specificity in detecting true templates of the SYBR assays.

All assays had highly reproducible results with intra- and inter-assay variability coefficient of variation of less than 5%. The primers were designed to be specific for a segment of the major capsid protein gene that previous studies have targeted with conventional PCR (Mao et al., 1997).

DNA from twenty-nine blood samples and thirty oral swabs were then screened for ranavirus. These samples were selected because they had previously been screened using conventional PCR and qPCR (Allender et al., 2011; Allender et al., 2012a). The results indicate that the SYBR-BTR quantitative PCR assay developed in this study was able to identify the same number of FV3-like virus positive samples. This may signify that this population of turtles indeed had a low prevalence of FV3-like virus (not infected), were not shedding ranavirus in the sample, or did not have enough viral copies to return a positive result. The epidemiology of ranavirus in turtles is not known, and therefore it is possible that when a susceptible individual is exposed to the virus, it either replicates to a level above 529,000 viral copies (conventional PCR level of detection) or does not replicate well. This would explain the results of conventional PCR and qPCR being similar. Regardless of the viral copies present in a clinical animal (as represented by these samples), early and subclinical detection will provide a valuable tool for control, monitoring, and management of this disease. To further elucidate the sensitivity of these assays compared with conventional PCR, it will be important to apply it to a set of experimental samples where the prevalence of disease is high. As such, the quantitative assays reported here may be more useful in detecting and tracking the persistence and development of disease as clinical animals are overwhelmed with similar viral loads.

Purity of DNA, determined by A260/A280 ratio, was within the ideal range (1.7 – 1.9) based on extraction guidelines for both whole blood and swab samples. However, the ratio was

non-normally distributed for swab samples and the range was greater than whole blood samples. When the purity of the sample is outside the ideal range, it can lead to a decrease in assay efficiency or an increase in non-specific parameters and inaccurate results. Therefore, purity should be evaluated prior to qPCR assay for all swab samples and samples outside the ideal range should be further purified before assaying.

The current study demonstrated that two SYBR green assays are reliable, specific, and sensitive for the detection of a gene segment of the MCP of frog virus 3.

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Table C.1. Primer and primer-probe sets used to amplify the major capsid portion of frog virus 3-like virus using conventional and quantitative PCR.

Assay	Primer name	Direction	Sequence 5’ . . . 3’	Expected amplicon size
qPCR	SYBR-BTR 1	Sense	GACGCTCTCCACAATGTTG	113
		Anti-sense	CCTGGTTGGTGCTCAAGAC	113
	SYBR-BTR 2	Sense	AACCAGGCGTTGAGGATG	110
		Anti-sense	TTCACCAAGCTGCCGTCT	110
Conventional PCR	SYBR-BTR	Sense	GACGCTCTCCACAATGTTG	113
		Anti-sense	CCTGGTTGGTGCTCAAGAC	113
	MCP 4 (Mao)		GACTTGGCCACTTATGAC	531
			GTCTCTGGAGAAGAAGAA	531

Table C.2. Intra- and inter-assay variability of two SYBR green and one TaqMan qPCR assays detecting frog virus 3 major capsid protein.

	Intra-assay				Inter-assay			
Viral Copy	CT mean	CT SD	CV	Melting point	CT mean	CT SD	CV	Melting point
SYBR-BTR 1								
5290000000	11.85	0.08	0.57%	84.83	11.86	0.07	0.52%	84.86
529000000	15.14	0.04	0.21%	84.99	15.14	0.03	0.19%	85.02
52900000	18.59	0.03	0.14%	85.11	18.62	0.05	0.24%	85.1
5290000	21.95	0.02	0.06%	85.23	21.96	0.02	0.10%	85.2
529000	24.94	0.03	0.09%	85.15	24.96	0.03	0.10%	85.13
52900	27.45	0.09	0.29%	84.99	27.47	0.08	0.26%	84.95
5290	28.81	0.12	0.36%	84.74	28.8	0.09	0.29%	84.79
529	29.48	0.03	0.08%	84.61	29.6	0.15	0.48%	84.56
52	29.72	0.10	0.29%	84.46	29.81	0.13	0.40%	84.49
NTC	31.35	0.13		83.00	31.48	0.23	0.68%	82.75
SYBR-BTR 2								
5290000000	11.76	0.02	0.14%	86.64	11.8	0.04	0.31%	86.62
529000000	15.03	0.02	0.09%	86.68	15.07	0.04	0.27%	86.68
52900000	18.54	0.02	0.10%	86.64	18.58	0.05	0.23%	86.62
5290000	21.85	0.02	0.08%	86.56	21.88	0.05	0.19%	86.56
529000	24.85	0.02	0.07%	86.40	24.9	0.05	0.20%	86.38
52900	27.34	0.05	0.17%	86.07	27.37	0.06	0.19%	86.1
5290	28.63	0.21	0.59%	85.74	28.2	1.15	3.66%	85.67
529	29.33	0.05	0.15%	85.85	29.48	0.16	0.51%	85.87
52	30.07	0.13	0.37%	85.95	30.11	0.15	0.46%	85.96
NTC	31.85	0.35		86.01	32.13	0.34	0.98%	85.94