IRIDOVIRUS INFECTIONS OF CAPTIVE AND FREE-RANGING CHELONIANS IN THE UNITED STATES

By

APRIL JOY JOHNSON

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2006

Copyright 2006

by

April Joy Johnson

ACKNOWLEDGMENTS

Funding for this work was provided in part by the University of Florida College of Veterinary Medicine Batchelor Foundation, grant No. D04Z0-11 from the Morris Animal Foundation, and a grant from the Disney Conservation Fund. I also thank the University of Florida, College of Veterinary Medicine for awarding me an Alumni Fellowship for tuition and stipend over the first three years.

I would like to thank the many people who helped collect and submit turtle and tortoise samples including Dr. William Belzer, Dr. Terry Norton, Jeffrey Spratt, Valorie Titus, Susan Seibert and Ben Atkinson. Also, I would like to thank all the pathologists who contributed to this work including Dr. Allan Pessier, Dr. Nancy Stedman, Dr. Robert Wagner and Dr. Jason Brooks. I thank Drs. Jerry Stanley and Kathy Goodblood for allowing me access to chelonian and amphibian populations at Buttermilk Hill Nature Sanctuary and to Drs. Mick Robinson and Ken Dodd for providing necropsy reports on archived cases. I would also like to thank the Mycoplasma Research Laboratory at the University of Florida including Dr. Mary Brown, Dr. Lori Wendland and Dina Demcovitz for providing access to their wild gopher tortoise plasma bank. I thank Yvonne Cates at the Zoological Society of San Diego for excellent histology support and Lynda Schneider from the University of Florida Electron Microscopy Core Laboratory. I also thank Dr. Harvey Ramirez, Rachelle Wright and Kevin Chadbourne with Animal Care Services for excellent care of the turtles and frogs.

Several people went above and beyond normal expectations in assisting with this research in various ways. I would especially like to thank Dr. Allan Pessier for all his time, patience and assistance with necropsies and histopathology as well as making me a better scientific writer. I thank Dr. Jim Wellehan for all his advice and assistance with molecular and laboratory techniques. I also am indebted to our laboratory technicians, Sylvia Tucker and April Childress. They both provided invaluable laboratory support and became great friends throughout my time at the University of Florida.

I thank my parents for their support and encouragement, without which I would never have made it to this point in my career. They have always believed in me and, although they might not always understand my unique interests, have never discouraged me from pursuing my goals. For that I will always be thankful.

And last, but far from least, I am extremely grateful to my supervisory committee members: Dr. Elliott Jacobson, Dr. David Bloom, Dr. Jack Gaskin, Dr. Jorge Hernandez and Dr. Gail Scherba. I thank them for the advice, support and encouragement they have given me since the beginning. I thank them for helping me develop my skills and for their confidence in my abilities. I have learned so much from them and have thoroughly enjoyed this opportunity to work with them.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

IRIDOVIRUS INFECTIONS OF CAPTIVE AND FREE-RANGING CHELONIANS IN THE UNITED STATES

By

April J. Johnson

December 2006

Chair: Elliott Jacobson
Major: Veterinary Medical Sciences

Iridoviruses of the genus *Ranavirus* are well known for causing mass mortality events of fish and amphibians with sporadic reports of infection in reptiles. The objective of this study was to characterize *Ranavirus* infections of chelonians. First, histopathologic and molecular investigations of naturally occurring infections in several species of chelonian were investigated. A virus isolate (BSTRV) obtained from a captive Burmese star tortoise (*Geochelone platynota*) was experimentally inoculated into western ornate box turtles (*Terrapene ornata ornata*) and red-eared sliders (*Trachemys scripta elegans*). Oral transmission failed to create illness, however five of six turtles inoculated intramuscularly developed clinical and histologic lesions consistent with naturally infected cases. Virus was re-isolated, fulfilling Koch's postulates and establishing BSTRV as a causative agent of disease and mortality in chelonians. Restriction enzyme analysis of this isolate with an isolate from a leopard frog (*Rana utricularia*) obtained at the site where the tortoise died was found to have identical restriction patterns suggesting

they are either the same or very closely related strains. This indicates that amphibians might serve as a source of infection for chelonians, or vice versa. BSTRV was also utilized as a coating antigen in the development of an indirect enzyme linked immunosorbent assay (ELISA). Plasma from a surviving pen-mate of the Burmese star tortoise served as a positive control for optimization. A seroprevalence study of 1000 banked free-ranging gopher tortoise plasma samples found that only 1.5% of tortoises were positive for exposure to the virus. The role of amphibians in the route of transmission of virus was assessed by experimentally inoculating leopard frogs, euthanizing them, homogenizing them, and feeding them to turtles via feeding tubes over a six-week period. All turtles failed to develop clinical signs or to produce anti-*Ranavirus* antibodies over three months. Lastly, the antiviral compound acyclovir was assessed at 0, 0.2, 1, 5, 10, and 25µg/ml for its ability to reduce or eliminate virus replication *in vitro* and to create cytotoxicity in *Terrapene* heart cells. No cytotoxicity was observed at any concentration. Increasing concentration found only a slight ten fold reduction in virus titer from $10^{4.8}$ to $10^{3.8}TCID_{50}$.

CHAPTER 1
INTRODUCTION

Chelonians are long-lived animals within the class *Reptilia*. The order *Chelonia*, also sometimes called Testudines, consists of two suborders and thirteen families of turtles and tortoises. Chelonians worldwide are experiencing dramatic declines. The 2006 International Union for Conservation of Nature (IUCN) Red List of Threatened Animals has listed 26 species as critically endangered, 45 as endangered and 58 as vulnerable of 295 species (43.7%). An additional 41 (13.9%) species are listed as near threatened. Other earlier estimates indicated about 50% of all taxa to be experiencing difficulties (Jacobson *et al.*, 1999). Reasons for declines include habitat fragmentation, increased collections for the food and pet market, change in vegetation, drought, and debilitating diseases (Jacobson *et al.*, 1999; Dodd, 2001). Chelonians have low fecundity and low juvenile survival rates, indicating that a loss of adult animals can have a significant impact on population survivability (Heppell, 1998). Native species of chelonians are experiencing similar declines. Two of the three tortoises within the U.S. [desert tortoise (*Gopherus agassizii*) and gopher tortoise (*G. polyphemus*)] are listed as vulnerable by the IUCN. Continuing decline and disappearance of box turtle (*Terrapene* spp) populations across the genus' entire range during the last century brought the 1995 listing of all box turtle species in appendices of The Convention on International Trade in Endangered Species (CITES) in an effort to slow their declines. Progress toward conserving these species is needed now before they become endangered.

Several important diseases have been identified in populations of wild and captive chelonians. Marine turtle fibropapillomatosis is a significant health problem affecting several species of marine turtles around the world (Herbst and Jacobson, 1995). Mycoplasmosis is a chronic infectious disease that has been seen in wild gopher tortoises in Florida and desert tortoises in the southwest deserts of the United States (Brown *et al.*, 2002). Tortoise herpesviruses have emerged as important pathogens of captive tortoises in the pet trade. Tortoise herpesvirus-1 is a causative agent of rhinitis-stomatitis complex in a variety of tortoise species (Origgi *et al.*, 2004) and tortoise herpesvirus-2 has also been seen associated with rhinitis and stomatitis in a captive desert tortoise (Johnson *et al.*, 2005). A study looking at all wild reptile cases submitted to the Wildlife Center of Virginia between 1991 and 2000 showed that 2% (n=694) of all cases were a result of infectious disease, although further characterization was not described. A total of 15 cases were infectious, of which 14 were eastern box turtles (*Terrapene carolina carolina*) and the other case was a rat snake. Two cases had respiratory tract infections, nine had conjunctivitis while the other four had both respiratory tract infections and conjunctivitis (Brown and Sleeman, 2002). This dissertation will demonstrate that *Ranavirus*es are also important emerging pathogens in wild and captive tortoises, and box turtles in the United States. This virus had previously been observed in a gopher tortoise (Westhouse *et al.*, 1996) and a box turtle (Mao *et al.*, 1997) in the United States. However, research presented here will show that it is likely responsible for other past and recent die-offs of box turtles in the eastern U.S. and may be a cause of unexplained deaths and population declines of gopher tortoises in Florida.

Iridoviruses are large double stranded cytoplasmic DNA viruses (Williams, 1996) that were first discovered in crane fly larvae in 1954 (Xeros, 1964) exhibiting a blue iridescence below the epidermis, which later led to the name iridescent virus or iridovirus (Williams and Smith, 1957). Only the insect viruses are known to create iridescence, whereas vertebrate iridoviruses do not. Iridoviruses are circularly permuted and terminally redundant (Goorha and Murti, 1982). Unlike poxviruses, which have a completely cytoplasmic site of replication (Schramm and Locker, 2005), iridoviruses require both the nucleus and cytoplasm for replication (Goorha *et al.*, 1978).

The family *Iridoviridae* consists of four genera. Two genera, *Chloriridovirus* and *Iridovirus*, infect insects. Viruses in the genus *Iridovirus* are typically smaller than those in the genus *Chloriridovirus*. Viruses in the genera *Lymphocystivirus* and *Ranavirus* are capable of infecting ectothermic vertebrates. Lymphocystiviruses infect fish, while ranaviruses have been shown to infect fish, amphibians and reptiles (Mao *et al.*, 1997). A group of unclassified erythrocytic viruses have also been attributed to the *Iridoviridae* family (Johnsrude *et al.*, 1997; Telford and Jacobson 1993); however further characterization is needed to establish the phylogenetic relationship of these viruses to the classified iridoviruses.

Frog virus 3 (FV3), the type species for the genus *Ranavirus*, was first isolated in 1966 from a renal carcinoma in a leopard frog (Granoff *et al.*, 1965), although it was subsequently determined that there was no association of the virus to the tumor (Granoff *et al.*, 1966). A *Ranavirus* also was recovered from bullfrog tadpoles manifesting a syndrome called tadpole edema virus (TEV) (Wolf *et al.*, 1968). In experimental studies, this virus was capable of infecting and causing significant mortality in Great Basin

spadefoot toads (*Scaphiopus hammondii intermontanus*), American toads (*Bufo americanus*), Fowler's toads (*Bufo woodhousii fowleri*), and bullfrogs (*Rana catesbeiana*) (Wolf *et al.*, 1968). A subsequent experimental study demonstrated Fowler's toads and the newt (*Diemictylus viridiscens*) to be susceptible to TEV, as well as two other isolates from frogs (LT1 and FV1) and two newt isolates (T8 and T15) (Clark *et al.*, 1969).

Significant research with amphibian iridoviruses did not make much progress until the early 1990s when worldwide declines in amphibians brought new interest regarding the role of these viruses in amphibian mortality events (Bradford, 1991; Speare and Smith, 1992; Fellers and Drost, 1993; Cunningham *et al.*, 1996; Fisher and Shaffer, 1996; Laurance *et al.*, 1996; Jancovich *et al.*, 1997; Bollinger *et al.*, 1999; Lips, 1999; Green *et al.*, 2002; Docherty *et al.*, 2003). In a study of sixty-four amphibian mortality and morbidity events, iridovirus was the most common cause of mortality (Green *et al.*, 2002). Late larval forms were more susceptible and epizootics were clearly associated with increased population densities. Affected salamanders exhibited problems with buoyancy, the inability to stay upright, swimming in circles, lethargy and red spots or swollen areas on the ventrum near the gills or hind limbs (Docherty *et al.*, 2003).

While iridoviruses have been occasionally reported as pathogens of reptiles (Marschang *et al.*, 2005; Hyatt *et al.*, 2002; Drury *et al.*, 2002; Johnsrude *et al.*, 1997), they have not received as much attention compared to iridovirus infection of amphibians. In chelonians, the first report of an iridovirus infection involved a captive Hermann's tortoise (*Testudo hermanni*) that died with necrotizing lesions in the liver, intestine and spleen (Heldstab and Bestetti, 1982). Several years later, an epidemic in a captive group of Hermann's tortoises was reported (Muller *et al.*, 1988). While two iridoviruses were

identified in an exotic tortoise (*Testudo horsfieldii*) and a box turtle (*Terrapene carolina*) in the U.S., no disease or pathology was mentioned (Mao *et al.*, 1997). The only report in a wild tortoise involved a gopher tortoise (*Gopher polyphemus*) in Florida that had signs of respiratory disease (Westhouse *et al.*, 1996). While viral particles were seen on electron microscopy, there was no attempt at virus isolation or molecular characterization. Recent isolates from two of seven Hermann's tortoises that died in a zoo in Switzerland were found by polymerase chain reaction (PCR) to have major capsid protein sequences closely related to FV3 (Marschang *et al.*, 1999). Around this time, iridovirus infections were documented in soft-shelled turtles (*Trionyx sinensis*) exhibiting cervical cutaneous erythema or "red-neck disease" at a turtle farm in China (Chen *et al.*, 1999). In experimental infections with the iridovirus isolated from the soft-shelled turtles, the virus was shown to be a causative agent of the "red neck" syndrome in young inoculated turtles. Most recently, *Ranavirus* infections were identified in a group of seven captive eastern box turtles in North Carolina in 2002 (DeVoe *et al.*, 2004). Six of these seven were wild caught with the most recent having been added 6 months prior to the outbreak. Clinical signs associated with *Ranavirus* infections in these box turtles were cutaneous abscessation, oral erosions or abscessation and respiratory distress (DeVoe *et al.*, 2004). One of seven affected turtles also showed unilateral conjunctivitis and cellulitis of the head and neck.

Ranaviruses are variably host specific and are widespread geographically (Chinchar, 2002, Daszak *et al.*, 1999). Virus can grow in multiple types of cell lines including fish, amphibian, reptilian, avian and mammalian, provided temperatures are conducive to growth (Chinchar, 2002). Evidence of viruses being capable of infecting

multiple species of animals has been demonstrated both naturally and experimentally. Inter-class infections with *Ranavirus* may occur among sympatric species in the wild, with fish or amphibians serving as the reservoir host (Mao *et al.*, 1999). Moody and Owens (1994) demonstrated an anuran virus, Bohle iridovirus, to be pathogenic for a fish, *Lates calcarifer*. Experimental transmission of Bohle iridovirus, a virus isolated from amphibians in Australia, was experimentally inoculated into six species of reptiles of which two turtle species, *Emydura krefftii* and *Elseya latisternum*, appeared to be susceptible showing increased mortality in inoculated hatchlings (Ariel, 1997). An insect iridovirus was isolated from a chameleon, two bearded dragons, and a frill-neck lizard (Just *et al.*, 2001). Thus, some of these viruses are very unusual in their ability to infect phylogenetically distinct lineages of vertebrates and invertebrates. However, other isolates have been shown to be very host specific and cannot be transmitted experimentally to other classes of animals (Jancovich *et al.*, 2001).

Natural transmission of iridoviruses has yet to be definitively identified, and may vary between genera or species of viruses. Experimental studies have shown that cannibalism of infected animals or ingestion of infected water may serve as a route of infection in amphibians (Jancovich *et al.*, 2001; Pearman *et al.*, 2004). Experimental infections of salamanders with a *Ranavirus* showed that both dose and host characteristics influenced the virulence of infection (Brunner *et al.*, 2005). The infection dose was positively correlated with mortality rate and inversely related to average survival times. Environmental temperatures have also been shown to significantly impact the percent mortality and time to death in salamanders experimentally infected with a *Ranavirus* (Rojas *et al.*, 2005), where salamanders infected at 18 and 10°C were more

likely to die than those exposed at 26°C. Two antimicrobial peptides, esculentin-2P and ranatuerin-2P, isolated from the skin of leopard frogs (*Rana pipiens*) are able to inactivate Frog Virus 3 in a dose dependent manner (Chinchar *et al.*, 2001) and may play a role in viral resistance.

The immune response of reptiles to iridoviruses has not been previously investigated; however, some research has been performed in this area for amphibians. Birds, reptiles and amphibians produce a low-molecular weight immunoglobulin called IgY (reviewed in Warr *et al.*, 1995). A study of 21 wild caught cane toads (*Bufo marinus*) in Townsville, Australia, found three toads to have anti-iridovirus antibodies cross-reactive against both epizootic haematopoetic necrosis virus and bohle iridovirus on enzyme linked immunosorbent assay (ELISA). Sera from positive toads or toads that had been exposed to an iridovirus showed positive to negative (P/N) ratios of 2.81, 2.91 and 3.4 compared to sera from naive toads, which ranged from 0.55 to 1.13 (Whittington *et al.*, 1997). Other studies in amphibians have focused on the African pipid frog, *Xenopus laevis*. One study experimentally infected adult frogs four weeks apart. Anti-FV3 IgY was detected in plasma one week following the second injection. This appearance of antibodies correlated to the time of viral clearance and the amelioration of clinical signs (anorexia and cutaneous erythema), suggesting a role of the adaptive immune system in clearing infection (Gantress *et al.*, 2003). This study also suggests the genotype of the MHC to play a significant role in the host susceptibility. Inbred *Xenopus* having a decreased MHC class I expression were more susceptible to infection, as were *Xenopus* larvae, which also lack MHC class I gene expression (Gantress *et al.*, 2003). Another study looking at antibody response to FV3 in *Xenopus* also showed that a second

exposure was necessary for developing a detectable response of IgY production (Maniero *et al.*, 2006). Antibodies were first detectable 10 days after a second exposure and levels plateaued at 14 days. IgM levels were never detectable even after three exposures.

Prevention of iridovirus infections has been researched primarily in fish. A vaccine against red seabream iridovirus (RSIV) has shown that genetic vaccines can be effective in protecting red seabream (*Pagrus major*) against experimental challenge with RSIV (Caipang *et al.*, 2006). Inoculation of juvenile red seabream with DNA plasmids encoding either the major capsid protein gene or an open reading frame encoding a transmembrane domain against RSIV showed upregulation of transcription of the MHC class I gene. Additionally, DNA vaccinated fish showed lower mortalities after subsequent exposure to RSIV than did non-vaccinated fish. Formalin-killed virus has also been shown to be effective in upregulating MHC class I transcripts (Nakajima *et al.*, 1997; Nakajima *et al.*, 1999). A field trial testing the efficacy of a formalin-killed virus showed a 49.3% decrease in mortality between vaccinated and control groups, and a statistically significant increase in size of fish in the vaccinated group (Nakajima *et al.*, 1999). A recent study evaluated an environmental contaminant and its potential to contribute to increased mortalities in iridovirus infected amphibians (Forson and Storfer, 2006). Somewhat surprisingly, the authors found that atrazine at moderate doses may reduce the efficacy of iridoviruses and, thus, protect animals exposed to both atrazine and iridovirus, resulting in decreased mortality rates. However, high levels of exposure to atrazine could result in decreased fitness. Salamanders were metamorphosed earlier and were smaller at metamorphosis than those exposed to no or moderate levels of atrazine (Forson and Storfer, 2006).

Treatment of iridovirus infections with antiviral drugs are not reported in the literature; however previous research has suggested that antiviral treatment with acyclovir might be successful. Acyclovir becomes activated by a viral gene product, an enzyme, thymidine kinase. Sequencing of the entire genomes of several isolates has identified open reading fames encoding putative thymidine kinase (TK) genes, and functionality was further studied in the Bohle iridovirus (BIV) (Coupar *et al.*, 2005). The BIV TK gene was inserted into a TK gene negative mutant vaccinia virus insertion plasmid, under the control of a vaccinia virus promoter and used to infect human 143B (TK-) cells. The investigators were able to show that this gene expressed a functional TK gene by rescuing of the TK negative mutant. Comparison of sequences of different iridovirus TK genes to other virus TK genes showed that unlike poxviruses, iridoviruses, similar to the herpesviruses, appear more closely related to the mitochondrial TKs and to cellular deoxycytidine kinases, whereas the TK gene sequence in other DNA viruses including poxviruses and African swine fever virus appear more closely related to the cellular TK genes (Coupar *et al.*, 2005). Thymidine kinase genes can activate nucleoside analogs such as acyclovir if the gene can recognize deoxycytidine as an alternative substrate. The ability of different iridovirus TKs to do this has not been evaluated. While TK genes appear well conserved among the different genera, they vary considerably between genera within the family, so each would need to be evaluated separately.

The research presented in the following chapters will investigate several aspects of *Ranavirus* infections in chelonians. Chapter 2 reports on molecular and histopathologic findings of natural cases of iridovirus infections in wild and captive chelonians in the United States. It describes several recent chelonian cases and two past die-offs of box

turtles dating back to 1998 and 1991 and demonstrates a new geographic range of infections from the northeast to Texas. Chapter 3 will describe the development and use of an indirect enzyme linked immunosorbent assay (ELISA) to evaluate the prevalence of iridovirus exposure in wild gopher tortoises. Chapter 4 will describe an experimental infection study fulfilling Koch's postulates, confirming that iridovirus is a primary pathogen of chelonians. Chapter 5 reports on a study investigating a possible route of transmission in which ingestion of infected amphibians could be a source of iridovirus in a natural setting. Chapter 6 will describe the use of an antiviral drug, acyclovir, at reducing replication of iridovirus in vitro. And lastly, chapter 7 will discuss overall conclusions and directions for future research.

CHAPTER 2
RANAVIRUS INFECTION OF FREE-RANGING AND CAPTIVE BOX TURTLES AND TORTOISES IN THE UNITED STATES

Introduction

It has been suggested that chelonians (turtles and tortoises) face a more serious threat than that posed by the well-publicized decline of amphibian populations (Klemens, 2000). Two thirds of all species of freshwater turtles and tortoises are currently listed as threatened on the IUCN Red List of Threatened Species (Turtle Conservation Fund, 2002). Chelonians have low fecundity, low juvenile survival rate, and a long adult lifespan; a life history strategy where loss of adult animals (such as loss by disease) has a significant impact on population recovery (Heppell, 1998). Emerging infectious diseases have been increasingly recognized as factors influencing wildlife health and populations (Harvell *et al*., 1999; Daszak *et al*., 2000). Although mycoplasmosis has been postulated to contribute to declines of some tortoise species (USFWS, 1994), the cause(s) of mass mortality events in wild chelonian populations often remain undetermined (Flanagan, 2000; Dodd, 2001).

Among the emerging diseases of wildlife, iridoviruses in the genus *Ranavirus*, are well known for causing mass mortality events of fish and amphibians (Langdon and Humphrey, 1987; Daszak *et al.*, 1999; Green *et al*., 2002). Iridovirus infections have also been sporadically described in reptiles including snakes (Hyatt *et al*., 2002), lizards (Marshang *et al*., 2005) and chelonians. In chelonians, iridovirus infections have been reported in captive Hermann's tortoises (*Testudo hermanni*) (Heldstab and Bestetti, 1982;

Muller *et al*., 1988; Marschang *et al*., 1999), farmed soft-shelled turtles (Chen *et al*., 1999) and captive eastern box turtles (DeVoe *et al*., 2004). Two ranaviruses identified in an exotic tortoise (*Testudo horsfieldii*) and a box turtle (*Terrapene carolina*) in the U.S., were found to be closely related to frog virus 3 (FV3) and designated as tortoise virus 5 and turtle virus 3, respectively (Mao *et al*., 1997). The only report of an iridovirus infection in a free-ranging chelonian involved a gopher tortoise (*Gopher polyphemus*) in Florida with signs of respiratory disease (Westhouse *et al*., 1996).

This report identifies *Ranavirus* infections in five recent chelonian deaths or mortality events from Georgia, Florida, New York, and Pennsylvania and in archived material recovered from previously unexplained mass mortality events in 1991 from Georgia and 1998 from Texas. This demonstrates a previously undescribed geographic extent of chelonian *Ranavirus* infections and suggests that ranaviruses may be more important pathogens of free-ranging chelonians than anticipated from previous reports. We also present molecular evidence for an identical or similar virus in frogs in the vicinity of two chelonian epizootics suggesting that amphibians could serve as reservoir hosts for chelonians.

Materials and Methods

Animals

Burmese star tortoises. Three female and two male captive Burmese star tortoises (*Geochelone platynota*) were kept in an outdoor enclosure at St Catherine's Island Wildlife Survival Center, Georgia (31°40'N/81°10'W) since April 2001. In early June 2003, two female and one male tortoise began showing clinical signs consisting of nasal discharge, conjunctivitis and severe subcutaneous edema of the neck (Fig. 2-1A). The tortoises were treated with antimicrobials, and were soaked daily for 90 minutes in warm

water. One female tortoise died three days after developing clinical signs and yellow white caseous plaques were observed on the tongue at necropsy. Oral antiviral therapy and intracoelomic fluids were then initiated in the surviving tortoises. Subsequently, six adult Southern leopard frogs (*Rana utricularia*) were sampled from within the tortoise pens and one was sampled from a pond nearby. One of the frogs from the pen was found moribund while others appeared healthy. Tissues from the dead tortoise and the leopard frogs were submitted for histopathology, polymerase chain reaction (PCR) for determining presence of certain DNA sequences of *Ranavirus* and *Herpesvirus*, and virus isolation.

Gopher tortoise. A wild gopher tortoise (*Gopherus polyphemus*) was found circling on a road in north central Florida (29°86'N/82°22'W) on 25 July 2003 and was brought to the University of Florida, College of Veterinary Medicine Zoological Medicine Service for evaluation and treatment. On presentation it exhibited palpebral swelling and ocular and nasal discharge (Fig 2-1B). The tortoise was treated with intracoelomic fluid twice daily, was allowed to soak in shallow warm water for 20 minutes a day and was started on antimicrobial therapy. The tortoise's condition continued to decline and it was euthanized with intravenous Beuthanasia-D solution (Schering-Plough Animal Health Corp., Kenilworth, NJ) on 29 July 2003. A complete necropsy was performed and tissues were submitted for histopathology, PCR and virus isolation.

Eastern box turtles. A 200 ha gated and fenced area within a private nature sanctuary in Venango County, Pennsylvania, (41º43'N/79º93'W) was created as a study site for relocated box turtles (*Terrapene carolina carolina*). All box turtles within the

study site were radiotelemetered and tracked regularly, and no box turtles were present at the site prior to the release of the relocated turtles. During the summer of 2003, there were 32 adult and 34 head-started juvenile turtles. Turtles were observed every five days to determine their health status and location. Fifteen of the 66 turtles (23%) died between 15 August and 20 November 2003. Many of the turtles were considered healthy approximately four to eight days prior to being found either moribund or dead. Nine of the turtles were found dead, while six were found moribund with palpebral edema, ocular discharge, and fluid draining from the mouth. Moribund turtles were taken out of the preserve, treated with an ophthalmic ointment in the eyes, soaked daily in warm water and given a temperature gradient. Two turtles were started on antimicrobials and one of the two also received a parasiticide. All moribund turtles died within hours to days after being found exhibiting signs of illness. In May 2004, two freshly dead green frog tadpoles (*Rana clamitans*) with marked cutaneous erythema were collected from a pond at the nature preserve. Tissues from five turtles collected in 2003 and from the tadpoles collected in 2004 were submitted for histopathology, PCR and virus isolation.

Two wild box turtles were found moribund with ocular discharge and swelling, as well as aural abscesses and yellow caseous plaques in the oral cavity in Suffolk County, New York (40º51'N/72º52'W) on 2 August 2005. They were observed near the edge of a drying pond that is utilized by many pond-breeding amphibians, including green frogs (*Rana clamitans*) and bullfrogs (*Rana catesbeiana*). These animals were taken to a local wildlife rehabilitator, where one died overnight. The carcass was then put in a freezer for later evaluation. The other had the aural abscesses drained and was treated with antimicrobials, but its health continued to decline until its death on 1 September 2005.

This turtle was put on ice and, along with the frozen specimen, sent to the University of Florida for histopathology, PCR and virus isolation.

Florida box turtle. A wild Florida box turtle (*Terrepene carolina bauri*), found in north central Florida (29°42'N/82°23'W) in October 2004, was submitted to the Zoological Medicine Service, College of Veterinary Medicine, University of Florida, for treatment. The box turtle exhibited palpebral edema, nasal and ocular discharge (Fig. 2-1C) and had yellow-white caseous plaques in the oral cavity (Fig. 2-1D). The turtle was administered fluid intracoelomically with B vitamins daily and was started on analgesics to alleviate pain. Due to failure to respond to therapy and a poor prognosis the turtle was euthanized three days after admission with intravenous Beuthanasia-D solution. A complete necropsy was performed and tissues submitted for histopathology, PCR and virus isolation.

Past mortality events. Tissues from two previous box turtle epizootics of undetermined etiology were examined. In July and August 1991 over thirty Eastern box turtles were found dead in or near water sources in Murray county (34°45'N/84°47'W), northwest Georgia (Dodd, 2001). Two moribund turtles were found exhibiting lethargy, ocular discharge and had caseous white plaques in the oral cavity. One turtle also had a subcutaneous abscess caudal to the left eye. Both turtles were submitted for necropsy. In 1998, several Eastern box turtles and other unspecified turtle species died suddenly in a private collection in Texas (Dodd, 2001). Archived paraffin blocks for histologic examination were obtained from two box turtles from the Georgia die off and one box turtle from Texas and re-evaluated using light and transmission electron microscopy.

Necropsy and Histopathology

At necropsy, tissues were collected from all major organ systems of the following tortoises and turtles: Burmese star tortoise (1), gopher tortoise (1), eastern box turtles [Pennsylvania (5), Georgia (2), New York (1), and Texas (1)] and Florida box turtle (1). Tissues were fixed in neutral buffered 10% formalin, dehydrated in graded alcohols, embedded in paraffin, sectioned at 6μm, and stained with hematoxylin and eosin. Tongue, liver and spleen of each animal were collected and frozen at –80°C for detecting DNA sequences of *Ranavirus* and *Herpesvirus* using PCR and virus isolation.

Nucleotide Amplification, Sequencing, and Sequence Analysis

DNA was extracted from chelonian and amphibian tissues and cell cultures used for virus isolation using the DNeasy kit (Qiagen, Valencia, CA, USA). Five 5μm thick paraffin embedded sections from box turtles from the 1991 and 1998 mortality events were extracted using the DNeasy kit following the protocol for paraffin embedded tissue. Sense primer (5'-GACTTGGCCACTTATCAC-3') and anti-sense primer (5'-GTCTCTGGAGAAGAAGAA-3') as previously described (Mao *et al*., 1997) were used to amplify approximately 500 base pairs of the *Ranavirus* major capsid protein gene. A 50μl reaction mixture was run which contained 4μl extracted DNA, 1 μM of each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 2.5 U of *Taq* DNA polymerase and PCR buffer containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ (Eppendorf, Westbury, New York, USA). The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid) with an initial denaturation at 94˚C for 2.5 min, followed by 25 cycles of denaturation at 94˚C for 30 sec.; annealing at 50˚C for 30 sec, extension at 72˚C for 30 sec., and a final extension step at 72˚C for 10 min as previously described

(Marschang *et al.*, 1999). The same extracted DNA from the chelonian tissues was evaluated by PCR for the presence of chelonian herpesvirus(es). A nested consensus PCR was performed as previously described to detect a portion of the herpesvirus DNA dependent DNA polymerase (VanDevanter *et al*., 1996).

Any PCR products were resolved in 1% agarose gels and bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Products were sequenced in both directions directly using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, New Jersey) and analyzed on ABI 377 automated DNA sequencers at the University of Florida's Sequencing Center.

Virus Isolation

Turtle heart cells [TH-1; American Type Cell Culture (ATCC), Manassas, VA] were seeded into 25 cm^2 flasks (Costar, Corning, NY, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, CA, USA), gentamicin (60 mg/liter) (Sigma, St. Louis, MO, USA), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300μg/liter) (Sigma) and cultured to confluency. A small piece of spleen or liver from each case was homogenized in separate 5ml tissue grinders containing DMEM. Part of each homogenate was applied to a flask of confluent monolayer of TH–1 cells while the other was passed through a 0.45μm filter (Costar) onto another flask of cells. Cells were incubated at 28º C. Flasks were observed daily for cytopathic effect (CPE).

Transmission Electron Microscopy

Second passage TH-1 cell monolayers (75mm^2 flasks) inoculated with first passage isolates from homogenates of kidney tissue collected at necropsy from the Burmese star tortoise and the gopher tortoise were examined by transmission electron microscopy (TEM). Cells were harvested 3 days after infection (2 days after observation of CPE) and centrifuged at 4,500 x *g* for ten minutes. Supernatant was discarded and the remaining pellet was suspended in Trump's fixative (4% paraformaldehyde, 1% gluteraldehyde). Cells were post-fixed in osmium tetroxide, dehydrated in graded alcohols and embedded in Spurr's resin. Tissues were viewed using a Hitachi H7000 transmission electron microscope at the University of Florida Electron Microscopy Laboratory.

Paraffin embedded spleen from a box turtle from the 1991 mass mortality event in Georgia and paraffin embedded trachea from a box turtle from Texas from the 1998 mass mortality event were deparaffinized in xylene, embedded in Spurr's resin, sectioned for TEM and examined as described for cell culture.

Skin from the necropsied Burmese star tortoise was placed in Trump's solution (McDowell and Trump, 1976) was submitted to the Athens Diagnostic Laboratory, University of Georgia for TEM. Tissue was post fixed in osmium tetroxide, dehydrated in graded alcohols and embedded in epoxy. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JSM-1210 transmission electron microscope.

Restriction Enzyme Analysis

Frog Virus 3 was obtained from ATCC and served as the positive control for comparative purposes with the viruses isolated in this study. Frog Virus 3 and second passage isolates of tissue homogenates from the Burmese star tortoise and Southern

leopard frog that were collected at the same site were inoculated onto TH-1 cells. Once CPE was observed, viral DNA was radiolabeled with [methyl-^{3}H] thymidine, extracted and digested with *Hind*III and *Xba*I endonucleases as previously described (Mao *et al*., 1999). Restricted DNA fragments were separated on a 0.7% agarose gel, after which the gel was impregnated with Enhance (Perkin Elmer, Wellesley, MA) according to the manufacturer's directions and the radiolabeled fragments were detected by fluorography.

Results

Necropsy and Histopathology

Histologic findings were similar in the Burmese star tortoise, Gopher tortoise and box turtles. Consistent lesions in all animals were necrotizing and ulcerative stomatitis or esophagitis, fibrinous and necrotizing splenitis, and multicentric fibrinoid vasculitis. Lesions in the oral cavity and esophagus were characterized by near diffuse mucosal erosion and ulceration with surfaces covered by a thick coagulum comprised of fibrin, degenerate heterophils, sloughed epithelial cells and bacterial colonies (Fig. 2-2). Lesions in the spleen consisted of disruption of the white and to a lesser degree, the surrounding red pulp by deposits of fibrin admixed with karyorrhectic debris, and infiltrates of small numbers of heterophils (Fig. 2-3). There was frequently mild to marked red pulp congestion and/or hemorrhage. Fibrinoid vasculitis with thrombosis was observed in splenic, sheathed capillaries (ellipsoids) in all animals and to varying degrees in other locations including oral mucosa, esophagus, stomach, intestine, skin, lung, heart, and liver. All animals had some degree of necrosis of hematopoietic tissue in the kidney, liver and bone marrow. Individual cases had multifocal necrotizing tracheitis (1/7), conjunctivitis (1/7) or gastritis (2/7). Rarely, basophilic intracytoplasmic inclusion bodies suggestive of iridovirus infection were observed within epithelial cells of the oral

mucosa, esophagus, stomach and trachea, and/or within endothelial cells, macrophages (Fig. 2-4) and hematopoietic progenitor cells. Inclusion bodies were observed in only 3 of 7 (Burmese star tortoise, eastern box turtle, and gopher tortoise) cases examined histologically.

Necrosis of hepatic and renal hematopoietic tissues with rare basophilic intracytoplasmic inclusion bodies consistent with iridovirus infection was observed in the moribund southern leopard frog from the Burmese star tortoise pen in Georgia and in one green frog tadpole from the site in Pennsylvania.

Archived tissues from the past two mortality events in Georgia and Texas showed similar histologic lesions as described above. These included fibrinous splenitis in all turtles as well as a necrotizing tracheitis with rare intracytoplasmic basophilic inclusion bodies in sloughed respiratory epithelial cells in the box turtle from Texas.

PCR and Sequence Analysis

PCR for the *Ranavirus* major capsid protein gene yielded DNA fragments approximately 500 base pairs in length (Fig. 2-5). After sequencing of the fragments and excluding the primer component, the sequences of the gopher tortoise, star tortoise, all box turtles, southern leopard frog and green frog shared 100% sequence identity. The sequences were compared to known sequences in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shiuoka, Japan) databases using TBLASTX (Altschul, *et al.*, 1997). TBLASTX results for the sequences all showed the highest score with FV3 capsid protein gene (GenBank accession # AF157769). Comparison of sequences showed that all isolates shared 100% sequence identity with FV3 across that portion of the major capsid protein gene. PCR of DNA extracted from paraffin

embedded sections of tissue from box turtles from the 1991 and 1998 die offs were negative. All tortoises and box turtles were negative by PCR for the presence of herpesvirus.

Virus Isolation

All TH-1 cells infected with tissue homogenates from the dead turtles and tortoises exhibited cytopathic effects (CPE) that consisted of cell rounding and lysis two to three days post infection. Flasks that contained both filtrated tissue homogenate as well as unfiltered tissue homogenate showed CPE. The Burmese star tortoise isolate was also passaged onto fathead minnow cells (ATCC), which subsequently exhibited similar CPE.

Transmission Electron Microscopy

Using TEM, the cell cultures infected with tissue homogenates from the Burmese star and gopher tortoises showed large numbers of icosahedral shaped viral particles that were consistent in size (approximately 130nm) and shape with an iridovirus (Fig. 2-6). Similar viral particles were observed within intracytoplasmic inclusion bodies in endothelial cells and macrophages of the skin in the Burmese star tortoise, within the cytoplasm of unidentified cells in the spleen of a 1991 box turtle from Georgia and within intracytoplasmic inclusion bodies of degenerate respiratory epithelial cells of the trachea in the 1998 box turtle from Texas (Fig. 2-7).

Restriction Enzyme Analysis

Repeated attempts failed to show discrete bands of the green frog tadpole after restriction with both enzymes, and thus, comparisons cannot be made with this isolate. Restriction with the *Hind*III enzyme demonstrated identical patterns between the FV3, the Burmese star tortoise and the leopard frog isolate collected at the same location as the star tortoise (Fig. 2-8A). The eastern box turtle isolate showed a slightly different

pattern. Restriction with *Xba*I found a similar result (Fig. 2-8B). FV3, the Burmese star tortoise and leopard frog isolates showed identical patterns, while the box turtle isolate was different.

Discussion

Emerging infectious diseases are those that have newly appeared in a population or have previously existed but are rapidly increasing in incidence or geographic range (Morse, 1995). The findings reported here suggest that iridovirus infections in chelonians fill this description, and are emerging pathogens of chelonians. Infections are being discovered in new populations of turtles, and the incidence is either increasing, or our ability to detect the disease in these animals is increasing. Infections in chelonians are more geographically widespread than has been previously documented. Previous reports from the United States had identified chelonian iridovirus infections in a wild gopher tortoise in Florida (Westhouse *et al.*, 1996), captive box turtles in North Carolina (DeVoe *et al.*, 2004) and in two chelonians where the location was not reported (Mao *et al.*, 1997). Here we have identified more infections than previously described within a two-year period in Georgia, Florida, Pennsylvania, and New York, indicating an increase in incidence of infection. Identification of iridovirus-like particles in the mortality event in 1998 expands the new geographic range further to include Texas. We also have described infection in a Burmese star tortoise, a species that has previously not been documented to be susceptible to iridovirus infections.

Reports of mortality events involving large numbers of box turtles and gopher tortoises have been documented in which etiologies were never definitively identified (Dodd, 2001; Rossell *et al.*, 2002; Seigel *et al*., 2003). This report demonstrates by re-

evaluation of archived samples that at least some of these mortality events (Dodd, 2001) have been infected with a virus(es) compatible with iridovirus and mortalities may have been caused by infection with these viruses. Polymerase chain reaction of these cases were negative, however it is unknown how long tissues had been fixed in formalin prior to being embedded into paraffin. Formalin fixation has been shown to degrade DNA and could result in false negative results (Tokuda *et al.*, 1990). Still, TEM of the same tissues showed virus particles consistent in size and shape with iridovirus.

The histologic lesions in the chelonians in this report were relatively non-specific and the intracytoplasmic basophilic inclusion bodies suggestive of iridovirus infection (Heldstab and Bestetti, 1982; Marschang *et al.*, 1999; Bollinger *et al.*, 1999; Docherty *et al.*, 2003) were absent or were rare and could easily be missed. Clinical and pathological differential diagnoses for the animals in this report prior to the demonstration of iridoviruses included chelonian herpesvirus infection (Johnson *et al.*, 2005) for the lesions of necrotizing stomatitis and septicemia for the fibrinous splenitis and multicentric vasculitis. While iridovirus infection should be considered in cases with lesions similar to those seen in the turtles and tortoises in this report, ancillary diagnostic tests, including viral isolation attempts and PCR for ranaviruses and herpesviruses, should be performed to confirm the diagnosis.

All chelonian and amphibian isolates in this study shared 100% sequence identity across a portion of the major capsid protein gene. The major capsid protein gene sequence is fairly conserved among iridoviruses, although one study has shown that it contains enough diversity to be able to distinguish closely related isolates (Tidona *et al.*, 1998). Mao *et al*. (1997) compared MCP sequences and restriction enzyme patterns of

whole genomic DNA of 10 vertebrate iridoviruses, including one virus isolated from a box turtle, Turtle Virus 3 (TV3). While the sequence obtained from a portion of the TV3 MCP gene indicated that it was identical to that of Frog Virus 3, a restriction enzyme analysis using *Hind*III and *Xba*I showed a different restriction pattern between the two isolates. Our study found a similar result. An isolate from a box turtle from the Pennsylvania die-off shared 100% sequence identity with FV3 across a portion of the MCP gene, however the whole viral genomic restriction enzyme analysis pattern differed from FV3, and showed a similar result to that of the box turtle isolate, TV3, reported by Mao *et al.* (1997). This suggests that the major capsid protein gene may be too conserved to determine if different animals are infected with the same virus.

An interesting and potentially significant finding was identical viruses, as determined by restriction enzyme analysis, in the Burmese star tortoise and a moribund southern leopard frog found within its pen. This suggests that both animals were infected with the same virus. Inter-class infections have been shown previously in a natural setting where sympatric species of amphibians and fish were infected with the same virus species (Mao *et al*., 1999) as well as through experimental transmission studies (Moody and Owens, 1994). There are several ways that chelonians and amphibians might be exposed to the same virus. Previous studies in salamanders have shown that transmission can occur through cannibalism of infected individuals (Jancovich *et al*., 2001; Pearman *et al*., 2004). Box turtles are omnivorous, and tortoises while normally herbivorous, may opportunistically feed on carrion. This was confirmed when animal caretakers at the site of the Burmese star tortoise death in Georgia observed a radiated tortoise (*Geochelone radiata*) and a Burmese black mountain tortoise (*Manouria emys phayrei*), both normally

considered herbivorous, eating dead amphibians in nearby pens. There could also be a common environmental source of virus, such as shared bodies of water. Iridoviruses are quite resistant and thought to be capable of persisting in water sources for extended periods of time (Daszak *et al*., 1999). Iridoviruses create systemic infections, and thus, a vector-borne route of transmission might also be a way that both amphibians and chelonians could become infected.

In summary, this report demonstrates that *Ranavirus* is an emerging pathogen of chelonians and suggest that amphibians might serve as a source of infection. This describes a new geographic range for chelonian iridovirus infections in the United States. Ranaviruses are considered a global threat to amphibian populations based on the lack of host specificity, high virulence and global distribution (Daszak *et al*., 1999) so they should likewise be considered a global threat to chelonian populations.

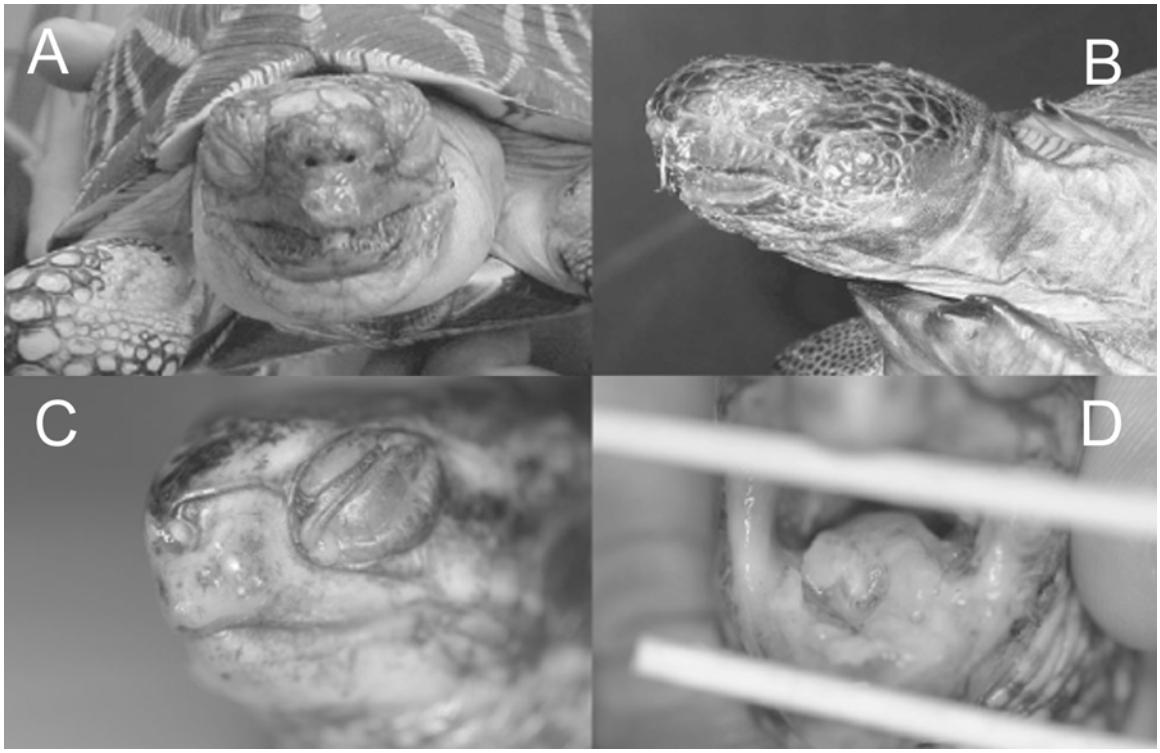


Fig. 2-1. Gross lesions associated with iridovirus infections in turtles and tortoises. A) Photograph of a Burmese star tortoise (*Geochelone platynota*) with nasal discharge and palpebral and cervical edema. B) Photograph of a wild gopher tortoise (*Gopherus polyphemus*) with nasal discharge and palpebral edema. C) Photograph of a wild Florida box turtle (*Terrapene carolina bauri*) with palpebral edema and ocular discharge. D) Photograph of caseous plaques in the oral cavity of a Florida box turtle.

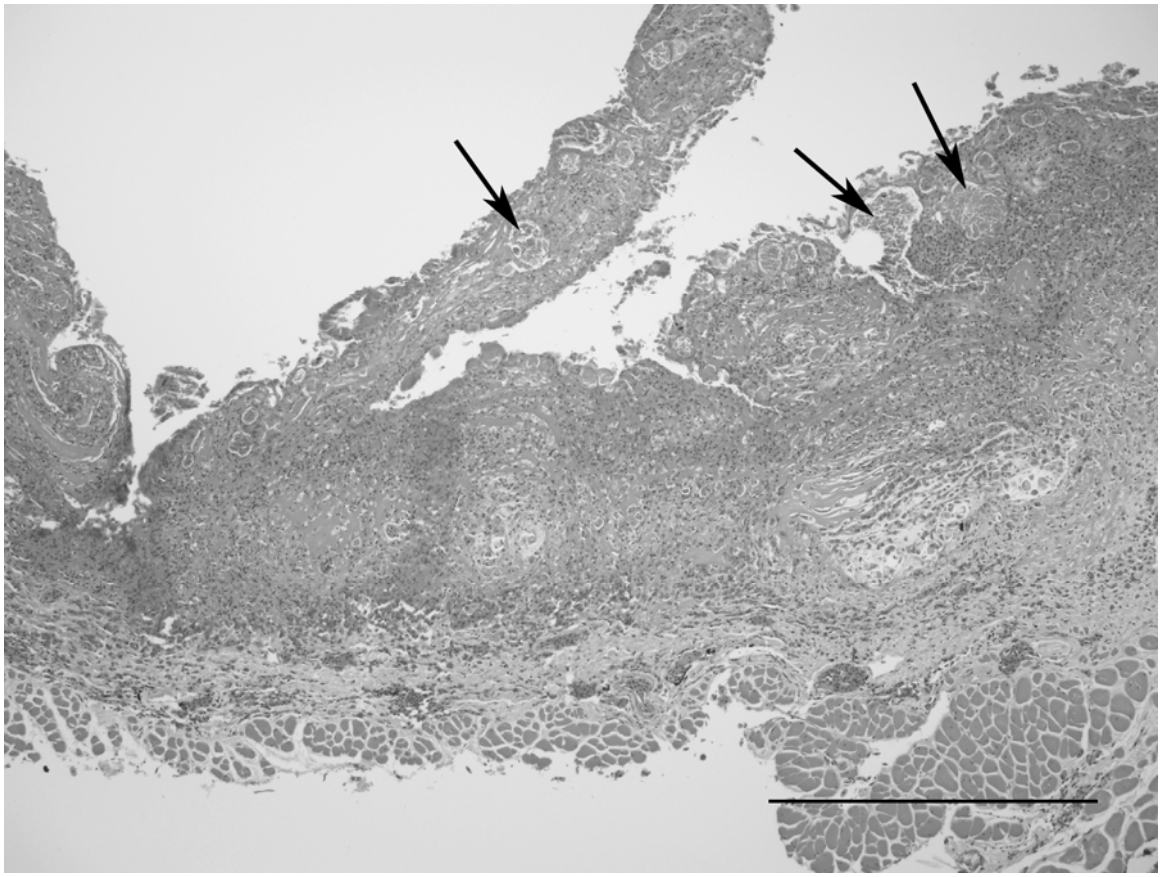


Fig. 2-2. Esophagus, eastern box turtle (*Terrapene carolina carolina*). There is diffuse necrosis and ulceration of the mucosa and replacement by fibrin, inflammatory cell infiltrates and superficial bacterial colonies (arrows). H&E stain.

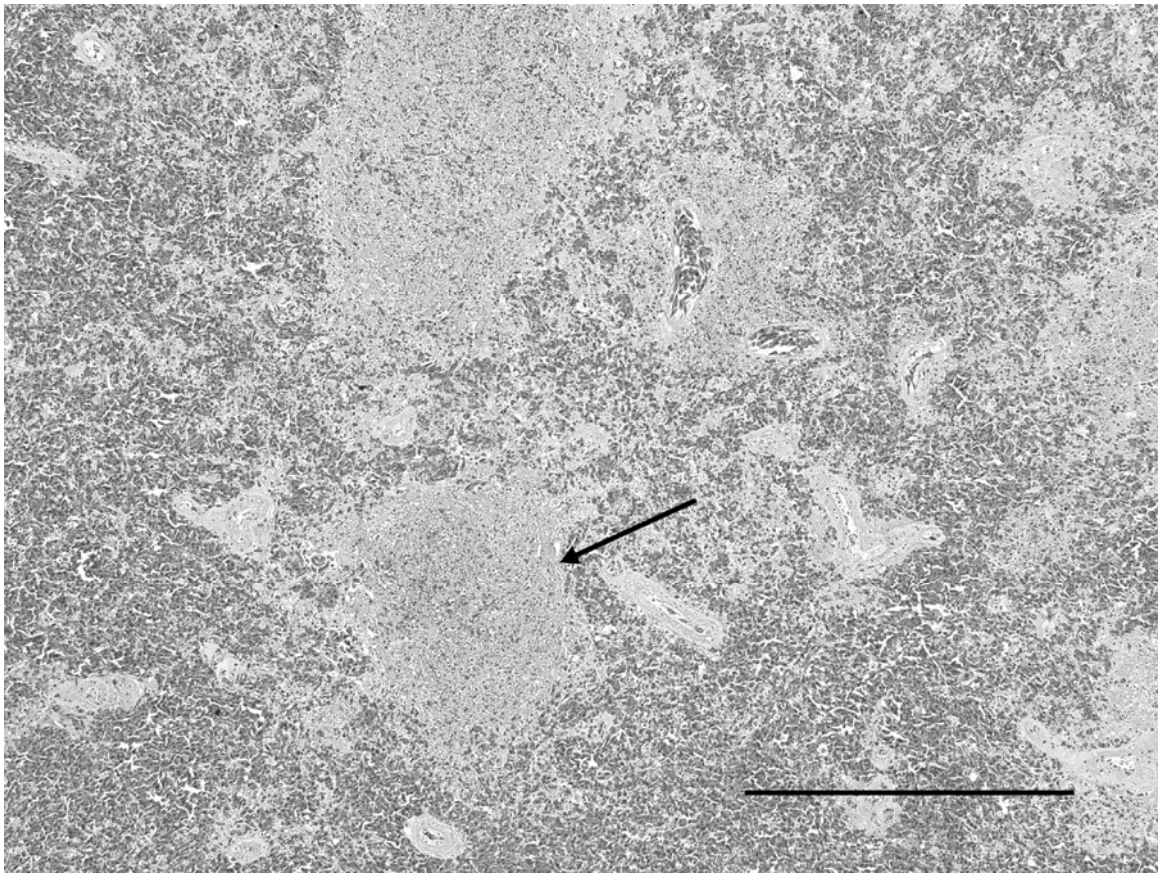


Fig. 2-3. Spleen, eastern box turtle (Terrapene carolina carolina). There is disruption of the white and red pulp with deposits of fibrin (arrow) admixed with karyorrhectic debris, and infiltrates of small numbers of heterophils. H&E stain.

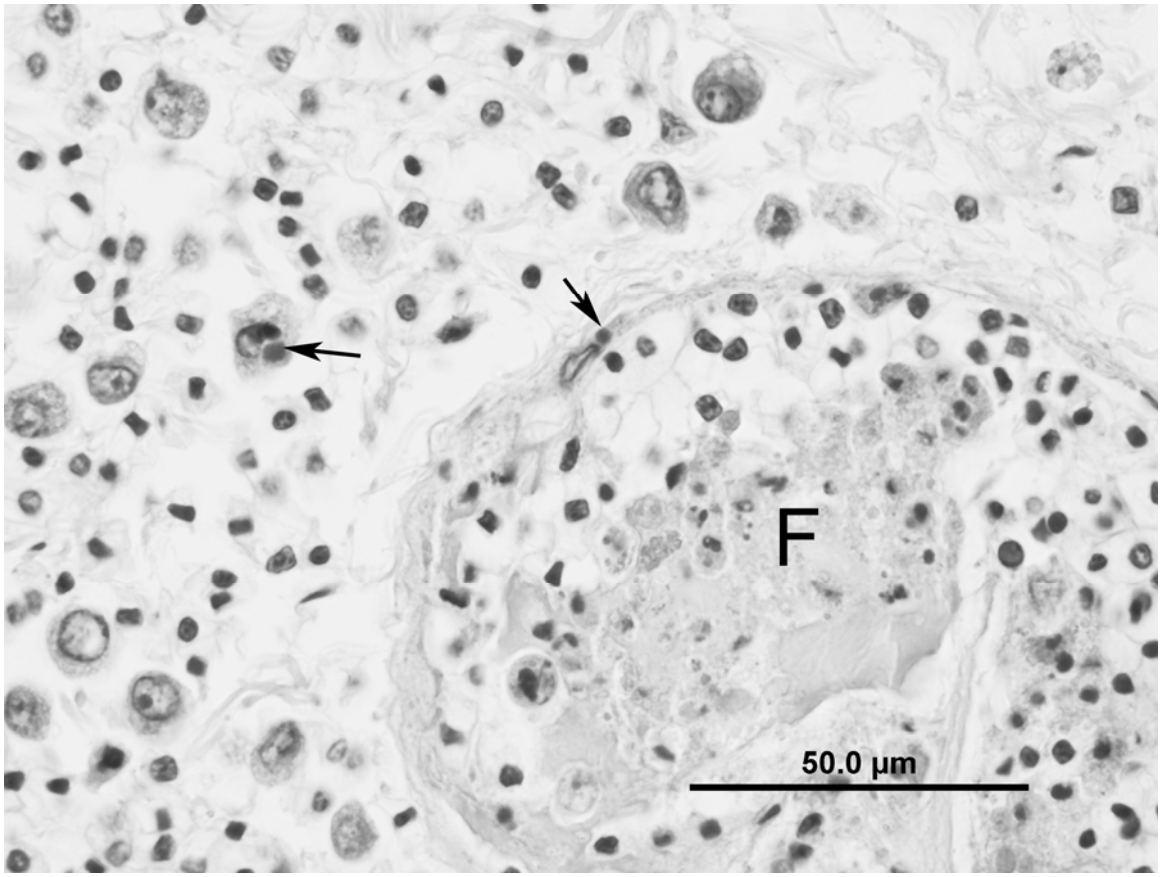


Fig. 2-4. Epicardium, Burmese star tortoise (*Geochelone platynota*). Arrows depict basophilic intracytoplasmic inclusion bodies in a macrophage and endothelial cell. The lumen of the blood vessel contains a fibrin thrombus (F). H&E stain.

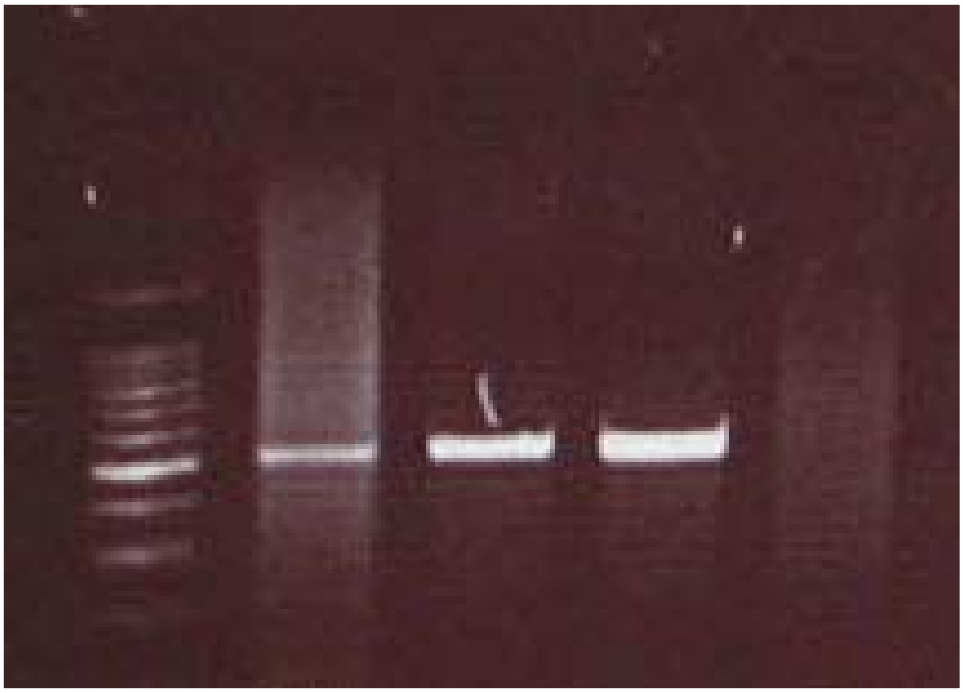


Fig. 2-5. Results of a polymerase chain reaction targeting approximately 500 bp of the major capsid protein gene. Lane 1 is a 100 bp ladder. The bright band represents a 500 bp fragment. Lane 2 and 3 are positive samples from an eastern box turtle (*Terrapene carolina carolina*) and a green frog tadpole (*Rana clamitans*). Lane 4 is a positive control sample from a Burmese star tortoise (*Geochelone platynota*) confirmed previously with nucleotide sequencing. Lane 5 is a negative control.

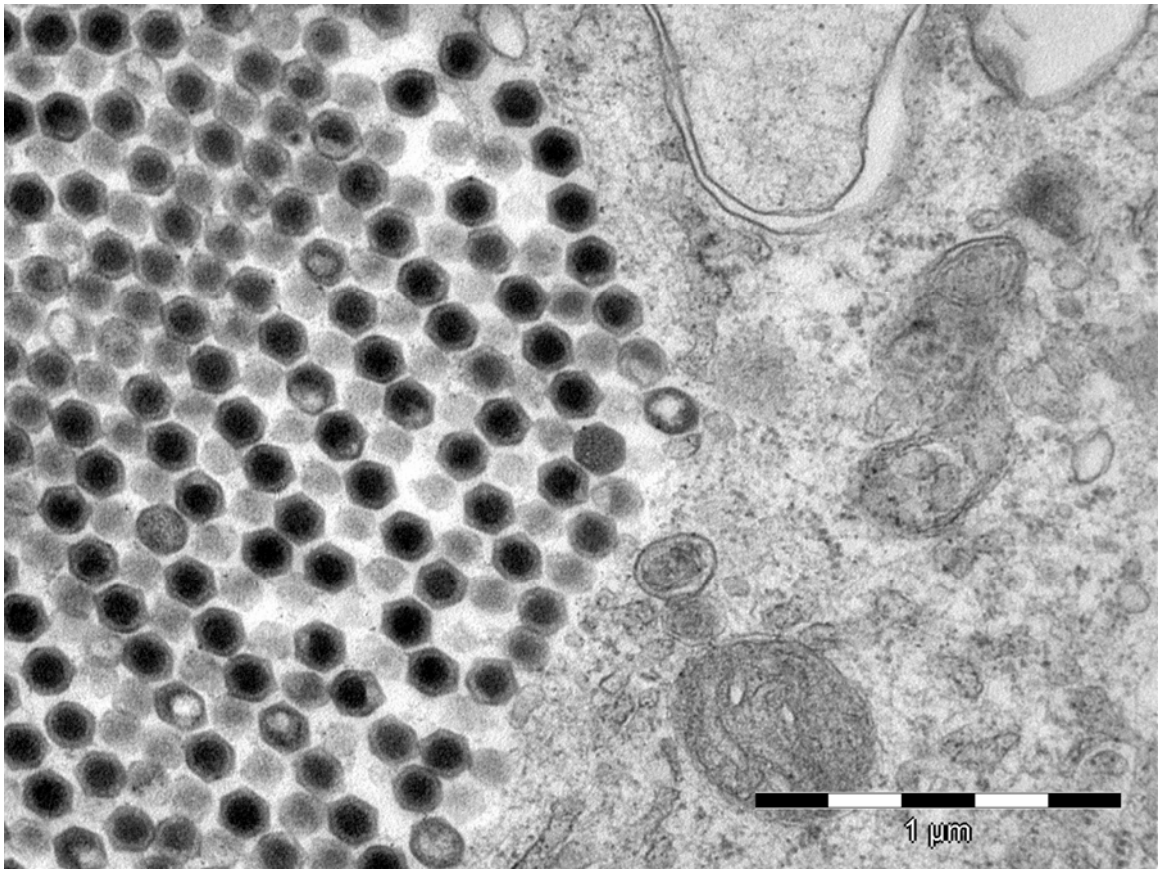


Fig. 2-6. Transmission electron photomicrograph of *Terrapene* heart cells inoculated with liver tissue from a Burmese star tortoise (*Geochelone platynota*) demonstrating cytoplasmic arrays of iridovirus-like particles.

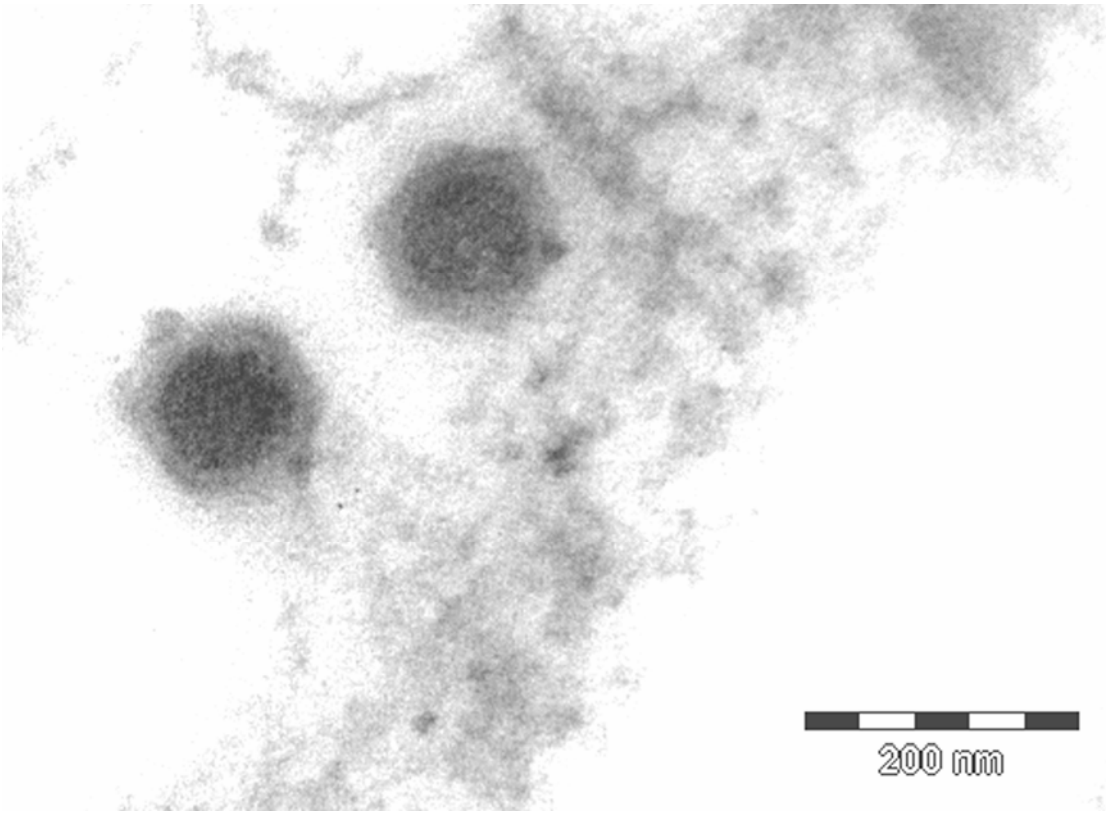


Fig. 2-7. Transmission electron photomicrograph of paraffin embedded spleen from a box turtle (*Terrapene carolina*) that died in 1991 in Georgia. There are icosahedral virus particles compatible with iridoviruses.

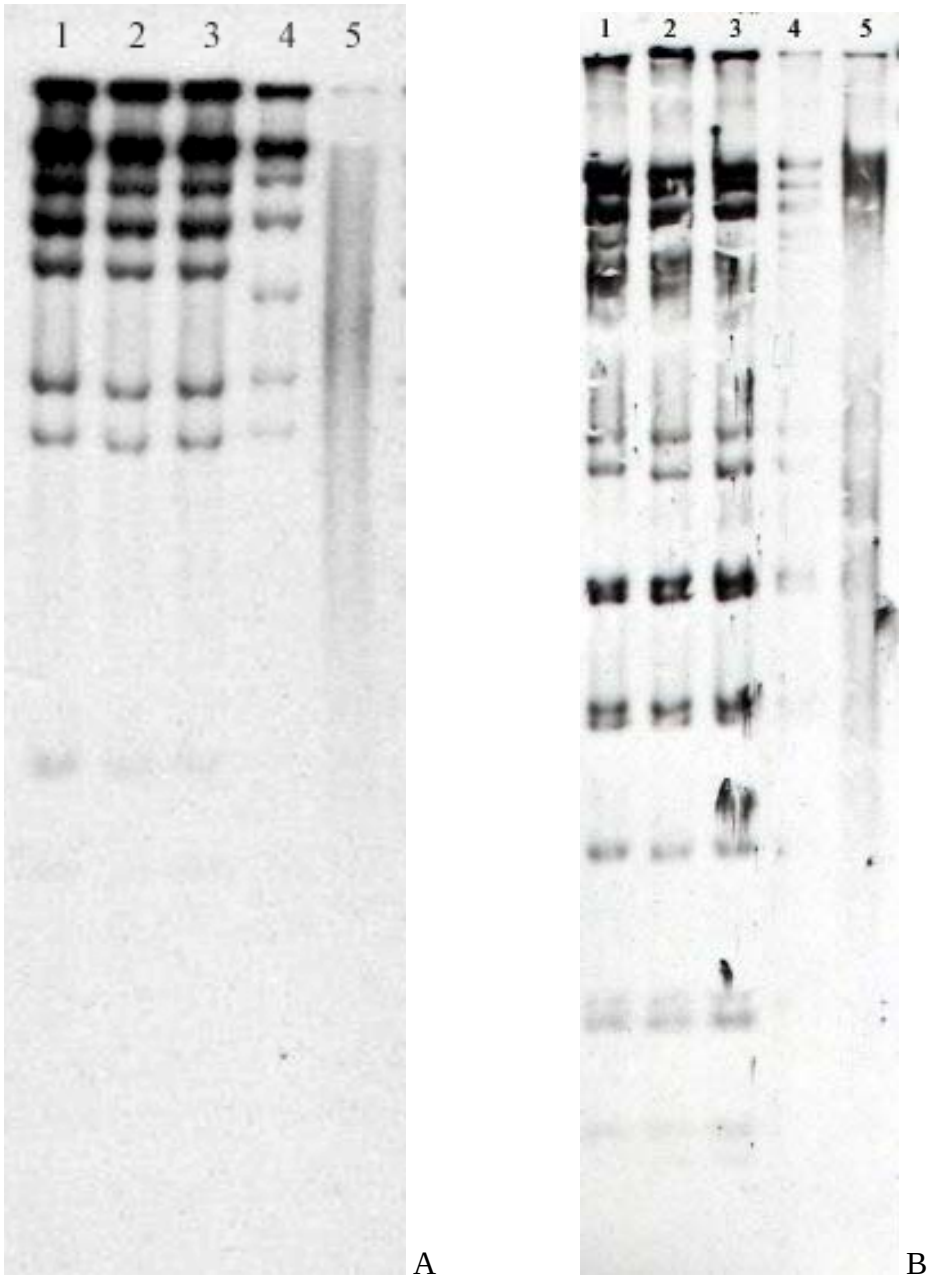


Fig. 2-8. *Hind*III and *Xba*I restriction enzyme pattern of five iridovirus isolates. A) *Hind*III restriction. Lane 1 is Frog Virus 3, the type species for the genus *Ranavirus*. Lane 2 is an isolate from a Burmese star tortoise (*Geochelone platynota*) and lane 3 is an isolate from a southern leopard frog (*Rana utricularia*) collected from the pen adjacent to the Burmese star tortoise. Lane 4 is an isolate from an eastern box turtle (*Terrapene carolina carolina*) from Pennsylvania and Lane 5 is a green frog tadpole (*Rana clamitans*) isolate from the same location as the box turtle. B) *Xba*I restriction pattern. Lanes are the same as those in Fig. A.

CHAPTER 3
DEVELOPMENT AND USE OF AN INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY FOR DETECTION OF IRIDOVIRUS EXPOSURE IN GOPHER TORTOISES (*Gopherus polyphemus*)

Introduction

Iridovirus infections of the genus *Ranavirus* have recently been identified in free-ranging and captive native chelonians from Florida, Georgia, New York, North Carolina, Tennessee, and Pennsylvania (Allender *et al.*, in press; DeVoe *et al.*, 2004; Johnson *et al.*, 2004). Species affected were Burmese star tortoises (*Geochelone platynota*), gopher tortoises (*Gopherus polyphemus*), eastern box turtles (*Terrapene carolina carolina*), and Florida box turtles (*Terrapene carolina bauri*). Evidence of iridovirus infection was also observed in archived material from previously unexplained mass mortality events of eastern box turtles (*Terrapene carolina carolina*) from Georgia in 1991 and Texas in 1998 (Dodd, 2001; Johnson *et al.,* 2004). Clinical signs associated with infections included those of upper respiratory tract disease including respiratory distress and nasal discharge, as well as oral ulceration, cutaneous abscessation, anorexia and lethargy (Westhouse *et al.,* 1996, DeVoe *et al.,* 2004). Consistent lesions in affected animals included necrotizing stomatitis and/or esophagitis, fibrinous and necrotizing splenitis, and multicentric fibrinoid vasculitis. Intracytoplasmic inclusion bodies were rarely observed in affected tissues. A portion of the major capsid protein (MCP) gene was sequenced from recent cases from Georgia, Florida and Pennsylvania and found to be identical across approximately 500 basepairs to each other and to Frog Virus 3 (FV3), the type species of the genus *Ranavirus* in the family Iridoviridae (Johnson *et al.,* 2004). Koch's

postulates were fulfilled by experimentally inoculating a tortoise *Ranavirus* isolate into red-eared sliders (Johnson *et al.,* unpublished findings). Three of four sliders developed severe clinical signs including anorexia (3/3), lethargy (3/3), oral plaques (1/3), nasal discharge (3/3), ocular discharge (3/3) and exophthalmus, conjunctivitis, and hyphema (1/3). Histologic changes were similar to those seen in naturally infected cases. Virus was isolated from tissues of each of the three turtles, fulfilling Koch's postulates and establishing iridovirus as a primary pathogen in chelonians.

Iridoviral infections are the most common cause of mortality events in amphibians in the United States (Green *et al.*, 2002). Iridoviruses are globally distributed and thus considered a threat to amphibian populations worldwide based on the lack of host specificity, high virulence and ubiquitous distribution (Daszak *et al.,* 1999). The geographic range of *Ranavirus* infections in chelonians in the U.S. has recently been found to be larger than previously known. Prior to 2003 only three cases of chelonian infections had been reported in the U.S.; however only one report included the location of the infected chelonian. A wild gopher tortoise from Florida was found to have iridovirus-like particles by transmission electron microscopy (Westhouse *et al.*, 1996). A box turtle and Russian tortoise (*Testudo horsfieldi*) isolate were described in another report (Mao *et al.,* 1997), but the location was not disclosed, and as Russian tortoises are not native to the United States, it is possible they were both kept as pets. Current published and unpublished reports now show a much larger geographic range with iridovirus infected chelonians identified from Texas to New York and Pennsylvania (Allender *et al.,* in press; DeVoe *et al.*, 2004; Johnson *et al.*, 2004). Therefore, it is reasonable to assume

that chelonians throughout the eastern United States can be exposed to iridoviruses; however, the prevalence rate of exposure has not been determined.

Serology can be a useful tool for detecting previous exposure to pathogens. Indirect enzyme linked immunosorbent assays (ELISA) have been used to detect exposure of various reptiles to specific pathogens (Schumacher *et al.,* 1993; Origgi *et al.,* 2001; Brown *et al.,* 2001; Jacobson *et al.,* 2005) and has been used to detect exposure of amphibians to iridovirus infections (Whittington *et al.*, 1997; Gantress *et al.,* 2003; Maniero *et al.,* 2006). To determine iridovirus exposure in wild gopher tortoises in the U.S., we developed an indirect ELISA using a previously developed mouse anti-desert tortoise IgY monoclonal antibody as the secondary antibody (Schumacher *et al.,* 1993). We also describe the results of a larger serological survey of wild gopher tortoises from various sites in Alabama, Florida, Georgia, Louisiana and Mississippi.

Materials and Methods

Virus

A *Ranavirus* isolated and partially characterized from a naturally infected Burmese star tortoise in Georgia (Johnson *et al.,* 2004), here termed BSTRV, 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 BSTRV isolate shared 100% sequence identity of approximately 500 basepairs with FV3. Transmission electron microscopy of BSTRV inoculated TH-1 cells showed virus particles in the cytoplasm of infected cells consistent in size and shape with iridoviruses. Restriction enzyme digests of BSTRV compared with FV3 showed identical restriction patterns using the enzymes *Hind*III and *Xba*I, indicating that BSTRV is either identical or very closely related to FV3.

Antigen Preparation

Two methods of antigen preparation were used. The first method of preparation was by sucrose gradient ultracentrifugation as previously described for epizootic haematopoietic necrosis virus of fish (Steiner *et al.*, 1991). *Terrapene* heart cells (TH-1) were acquired from the American Type Culture Collection (ATCC-CCL 50; Rockville, MD) and grown to confluency in 225cm^2 tissue flasks (Costar, Corning, NY). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60mg/liter; Sigma, St. Louis, MO), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300µg/liter; Sigma). Cells were inoculated with a fourth passage of BSTRV and incubated at 28ºC in the presence of 5% CO_2. When cytopathic effects (CPE) were observed in over 70% of cells, consisting of cell rounding and detachment from the flask, the flasks were frozen and thawed 3 times with vigorous vortexing before each freeze. Supernatant was transferred to 15ml tubes and clarified by low speed centrifugation at 4,500x*g* for 15 minutes. Supernatant was then decanted into two sterile 1-liter bottles and stored at 4°C until 1.5 liters of supernatant were obtained. Virus was then pelleted at 10,000x*g* for 8 hours at 4°C and the supernatant was discarded. Pellets were resuspended in the residual media, divided into four equal parts and overlayed on four 15-60% (w/v) sucrose gradients. Gradients were then ultracentrifuged at 150,000x*g* for 45 minutes at 4°C. Bands of purified virus were collected by fractionation and diluted in Tris-HCl (pH 8.0) until sucrose was less than 20%. Bands in Tris-HCl were layered onto four 5ml 20% (w/v) sucrose cushions and ultracentrifuged at 80,000x*g* for one hour at 4°C. Sucrose was next decanted and pellets were resuspended in 200µl Tris-

HCl and stored at -80°C. Purity was assessed with negative staining electron microscopy, protein assay (Biorad, Hercules, CA), PCR and ELISA.

The second way antigen was prepared was by creating a cell lysate from virus infected cells. Virus was inoculated onto TH-1 cell monolayers as described above in 225 cm^2 flasks. Flasks were scraped when flasks exhibited 100% CPE. 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 transferred to 15ml tubes and centrifuged at 4,500x*g* for 30 minutes. Supernatant was then discarded and the cell pellets were resuspended in residual media, and then frozen and thawed three times. Tubes were vortexed before and after each freeze cycle and following the final thaw, were centrifuged again at 4,500x*g* for 30 minutes. Supernatant was then transferred to a 4ml sterile cryotube. A protein assay was performed to determine the final protein concentration of the antigen. PCR was performed to confirm the presence of viral DNA.

Positive and Negative Reference Plasma

In July of 2003, three of five captive Burmese star tortoises became ill with clinical signs consisting of nasal discharge, conjunctivitis and cervical subcutaneous edema. One of the three tortoises died and histologic and molecular investigations demonstrated the presence of iridovirus in various tissues. Surviving tortoises were treated with supportive care and all four tortoises survived. Plasma was collected at the time of infection during July and then again in September 2003. Plasma from one of these tortoises was used as the positive control in development of the ELISA. Plasma

was collected from Burmese star tortoises from a zoological collection with no known history of disease to serve as negative reference plasma for the ELISA.

ELISA Procedure

A checkerboard optimization strategy was used to determine the optimum concentrations of both antigen and plasma to be used in the ELISA. Antigen concentrations evaluated were 1:100, 1:250, 1:500, and 1:1000. Plasma concentrations evaluated were two-fold serial dilutions from 1:50 to 1:1600. The following procedure was found to be optimal utilizing the crude cell lysate antigen. Each well of a high protein binding 96 well microplate (Maxisorp F96; Nunc, Kamstrup, Denmark) was coated with 50µl of infected or uninfected cell lysate diluted to 1:100 in 0.01M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl and 0.02% sodium azide (PBS/Az). Plates were then incubated overnight at 4°C. Antigen was then aspirated off and wells were washed four times with in ELISA wash buffer (PBS/Az with 0.05% Tween 20). This washing process was performed between each of the following steps. Wells were then blocked against non-specific binding with 300µl of Superblock blocking buffer by Pierce (Rockford, IL) for one hour at room temperature (RT). Each remaining step was incubated for 1hr at RT. Plasma samples diluted 1:100 in blocking buffer were added at 50µl volumes to wells in triplicate. One well was coated with uninfected cell lysate while the other two wells were coated with infected cell lysate. Next, a biotin-conjugated monoclonal antibody produced against the desert tortoise IgY light chain and previously used for detecting anti-mycoplasma antibodies in desert tortoises (Schumacher *et al.*, 1993) was diluted to a final concentration of 0.5µg/ml in PBS/Az and added to each well in 50µl volumes. Alkaline phosphatase-conjugated streptavidin (Zymed Laboratories,

Inc., San Francisco, California) was then applied to each well at 50µl of a 1:5000 dilution in PBS/Az. Next, the ELISA was developed with 100µl per well of a 1.0mg/ml *P*-nitrophenyl phosphate prepared in 0.01M sodium bicarbonate buffer containing 2mM $MgCl_2$ and plates were stored in the dark. The absorbance of each well was read at A_{405} using a StatFax 3200 microplate reader (Awareness Technology, Palm City, Florida) at 30 minutes.

Each plasma sample was read in triplicate. Plasma was placed on one well originally coated with uninfected cell lysate and on two wells coated with infected cell lysate. The average absorbance reading of the two wells coated with infected cell lysate was calculated and the positive/negative (P/N) ratio value of each sample was determined by dividing the mean absorbance of the duplicate average by the absorbance reading of the well from the uninfected cell lysate. This subtracts out any background noise, or non-specific binding that may have occurred as a result of using the *Terrapene* heart cell line. The cut-off value for a positive test result was made by adding three times the standard deviation of the mean P/N ratio to the mean (Crowther, 2001).

Experimentally Inoculated Turtles

Although iridovirus infection was confirmed in the Burmese star tortoise that died, it was not confirmed in any of its pen-mates. So while we assumed the other clinically ill tortoises were exposed, there was no way to definitively know. To develop known positive and negative samples for use in developing the ELISA and to validate the test by detection of seroconversion, ten red-eared sliders (*Trachemys scripta elegans*) ranging in weight from 775 to 1050g were obtained from a reptile dealer and allowed to acclimate in an animal care facility room at the University of Florida for three weeks.

This study was performed under the approval of the Institutional Animal Care and Use Committee at the University of Florida.

Oral and cloacal swabs were collected from turtles and ran by PCR as previously described (Mao *et al.*, 1997) for the presence of *Ranavirus* DNA sequences to determine current infection status. Plasma was collected and tested by the following ELISA to determine presence of anti-*Ranavirus* antibodies at the time of arrival, which would indicate previous exposure. Turtles were randomly assigned to one of three groups. Group 1 turtles (Nos. 13, 14, 16 and 20) received 1ml of a virus infected crude cell lysate prepared as described above and diluted to a final concentration of 10^2 $TCID_{50}$/ml orally (PO) by metal gavage feeding tube placed into the distal esophagus. Group 2 turtles (Nos. 12, 15, 17, 18) received the same dose of virus intramsucularly (IM), half the dose in the right and half the dose in the left pectoral muscles. The two other turtles were assigned to a control route of inoculation. Turtle 11 was mock inoculated with the same volume of an uninfected cell lysate by PO while turtle 19 received an uninfected cell lysate by IM. Plasma samples were collected weekly for five months from each turtle to attempt to detect the production of antibodies. Turtles were euthanized at five months, or when severe clinical signs of disease appeared including any of the following: severe lethargy, subcutaneous edema, nasal or ocular discharge, oral plaques, or hyphema. Tissues were collected at necropsy for PCR, histopathology and virus isolation.

Reproducibility

Intra-assay and inter-assay reproducibility were determined by performing two precision runs. The positive and negative reference plasma samples, used to optimize the test, were used in each assay. Intra-assay reproducibility was determined by running the positive and negative sample multiple times on the same plate. Each sample is run

multiple times on one well of uninfected cell lysate and two wells of infected cell lysate. This resulted in 64 readings for each sample on the infected cell lysate and 32 on the uninfected cell lysate. Inter-assay reproducibility was determined by using the values of the reference plasma results used as controls in running up the wild gopher tortoise samples from multiple dates and multiple plates. The mean A_{405}, the standard deviation (SD) and the coefficient of variation (CV) for the intra- and inter-assay reproducibility were calculated using the optimized ELISA conditions.

Protein Expression and Immunoblotting

The positive control plasma was tested for its ability to detect viral proteins in a western blot using infected and uninfected TH-1 cell lysates as antigen. Four four-fold dilutions of infected and uninfected cellular lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions along with broad range molecular weight markers. The separated proteins were transferred onto 0.2µm nitrocellulose membranes (Biorad, Hercules, CA) by standard methods (Harlow and Lane, 1988). Membranes were then rinsed in water for five minutes in preparation for coomassie blue staining or in tris buffered saline containing 0.5% Tween20 (TTBS, pH 7.5) for twenty minutes prior to immunoblotting. Membranes were then stained in coomassie blue stain for 90 minutes, followed by water for 30 minutes to destain to view differential protein profiles between infected and uninfected cell lysates. After rinsing in TTBS, membranes for immunoblotting were then blocked with Superblock blocking buffer in phosphate buffered saline (Pierce) for one hour. Blocking buffer was then removed and plasma samples diluted 1:2000 in blocking buffer were added to the membranes. After one hour, the membranes were washed with TTBS for 30 minutes and the monoclonal antibody was added, diluted 1:10,000 in

blocking buffer for one hour. Again the membranes were washed and AP streptavidin was added, diluted 1:5,000 in phosphate buffered saline for one hour. Membranes were washed as previously, and the membranes were developed in substrate buffer (0.1M Tris-HCl, 1mM $MgCl_2$) containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) (Biorad). The reaction was stopped by removing the NBT-BCIP solution and adding deionized water. Membranes were allowed to air dry.

Wild Gopher Tortoises Samples with Unknown Exposure

Plasma samples from 1000 wild gopher tortoises (*Gopherus polyphemus*) from Florida, Georgia, Alabama, Louisiana and Mississippi were obtained from samples submitted to the Mycoplasma Research Laboratory at the University of Florida from 2002 to 2006. County data was recorded when available, although it was not available in 68 cases. Samples were further subdivided by region of the state including central, central east, central west, north central, north east, north west, south east, and south west. Results were also compared according to state.

Results

Antigen Preparation

Two diffuse bands were observed on the sucrose gradients and each band was separated into two samples when placed on the sucrose cushion for pelleting. This resulted in four stocks of purified virus (two low bands - 1A and 1B and two higher bands 2A and 2B). Ten microliters of each stock was submitted for negative staining electron microscopy (EM). While virus particles were observed (Fig. 3-1), they were difficult to find by EM. A protein assay of each sample indicated protein concentrations of 1000 (1A), 375 (1B), 625 (2A) and 500 μg/ml (2B). Polymerase chain reaction of

each sample resulted in strong positive signals. Use of the purified antigen in an ELISA indicated approximately 37.5μg/ml of the purified virus was necessary for coating wells to obtain the best results. Because such a small volume of purified iridovirus resulted from the sucrose gradient purification protocol, and such a large volume was needed for use in the ELISA, it was decided that a crude cell lysate would be used for detecting seroconversion in the transmission studies and for the seroepidemiology study.

ELISA Parameters

Checkerboard optimization found that diluting the antigen 1:100 and diluting plasma samples also at 1:100 gave the largest difference between P/N ratios of positive and negative reference samples (Fig. 3-2). Plasma cut off values were determined after running the wild gopher tortoise samples. The frequency distribution was determined in tenth increments from 0.5 to 4.1 and the P/N ratios of the samples were determined to have a normal distribution, skewed to the right (Fig. 3-3). Because the data was normally distributed, the mean P/N ratio of samples (1.078), plus three times the standard deviation (0.379) was used to determine the positive cut-off value (2.2). P/N ratios from each sample were plotted on a graph with the sample numbers on the x-axis in increasing order and the P/N ratio values on the y-axis (Fig. 3-4). This plot shows a gradual increase in P/N ratios followed by a sharp increase around a P/N ratio of about 2.0, confirming that our cut off value of 2.2 to be a reasonable value.

Experimentally Inoculated Turtles

One IM inoculated turtle (No. 15) became extremely lethargic and died 24 days post-inoculation. Cloacal swabs taken on turtle 15 were positive by PCR and sequencing for the presence of iridovirus starting 8 days before the turtle died. Oral swabs were positive the day before the turtle died. Other than lethargy, no clinical signs were

observed in this turtle such as stomatitis, conjunctivitis or nasal discharge. Histologic changes were consistent with those seen in naturally and experimentally inoculated turtles (DeVoe *et al.*, Johnson *et al.*, unpublished data). Briefly, there was a fibrinoid vasculitis in the spleen with multifocal infiltrates of low numbers of heterophils and scattered free brown pigment granules (presumptively from disrupted melanomacrophage centers). There were occasional lumenal fibrin thrombi with admixed heterophils and karyorrhectic debris. No other turtles died during the five-month course of the study.

Plasma samples collected weekly over the five-month period failed to detect seroconversion in all of the mock inoculated (Fig. 3-5A) turtles and turtles orally inoculated with *Ranavirus* (Fig. 3-5B). One IM inoculated turtle (No. 12) had an increasing P/N ratio trend, but only twice did values exceed a ratio of 2, which occurred on weeks 8 and 18 (Fig. 3-5C). All other turtles remained well below the positive P/N ratio cut off value of 2.2.

Reproducibility

The mean A_{405}, SD and CV values for the intra- and inter-assay precision runs are shown in Table 1. Ideally, CV values should be less than 15% (Crowther, 2001). Three of the CV values in the precision runs are >15% indicating that some variability still exists in this assay.

Protein Expression and Immunoblotting

Coomassie blue staining of proteins from infected cell lysates and uninfected cell lysates showed a different pattern of protein expression (Fig. 3-6). Immunoblotting of infected and uninfected cell lysate showed a marked increase in binding to proteins in the infected cell lysate and very weak binding to proteins in the uninfected cell lysate (Fig. 3-7). Strong signals were seen on virus-infected cells at approximately 125 kDa, and 78

kDa with weaker signals seen at 70, 65, and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the uninfected cells.

Wild Gopher Tortoises Samples with Unknown Exposure

Of 1000 gopher tortoise plasma samples assayed, 15 (1.5%) were positive with a P/N ratio >2.2 (Table 2). Eight seropositive tortoises came from five counties in Florida including Lake, St. Lucie, Broward, Palm Beach and Martin (Fig. 3-8). While seropositive tortoises represent three regions including central, central east, and southeast, four of the five counties are clustered closely together in the south including Palm Beach, Broward, Martin, and St. Lucie. The remaining seven seropositive tortoises were located in Baker, Georgia, a county in the southwest corner of the state. Prevalence of seropositive tortoises by county was quite variable (Table 3). Approximately 3% of tortoises sampled in Lake county were positive (n=99), 2.9% in St. Lucie (n=35), 10% in Broward (n=10), 3.4% in Palm Beach (n=58), and 6.2% in Martin county (n=16). Of tortoises tested from Baker, GA 6.5% were positive (n=113). By state, Florida had an overall prevalence of 1.2% (n=658) while Georgia had a prevalence of 3.1% (n=225) (Table 3). All tortoises tested from Alabama, Mississippi and Louisiana were negative.

Discussion

Iridoviruses of the genus *Ranavirus* are emerging as important pathogens of chelonians. Previous reports have established their presence in Florida (Westhouse *et al.*, 1996) and in North Carolina (DeVoe *et al.,* 2004). Newer unpublished findings indicate a much larger range of infection including reports from Texas, Georgia, New York and Pennsylvania (Johnson *et al.,* 2004). The duration of illness can be rather short (Johnson *et al.*, unpublished findings), making it difficult to observe symptomatic tortoises in wild populations. Therefore, we developed an indirect ELISA to detect anti-*Ranavirus*

antibodies and performed a cross-sectional survey of gopher tortoises utilizing banked plasma samples to attempt to determine the prevalence of exposure of free-ranging chelonians to iridoviruses.

When developing a new assay, it is ideal to have another assay against which to compare results. With a reference assay, the sensitivity and specificity of the new assay can be evaluated, and thus the level of confidence one has in its ability to detect what you want it to detect. Unfortunately, this was not possible with this pathogen. Detectable neutralizing antibodies are not usually found in naturally or experimentally infected animals (reviewed by Whittington *et al.,* 1997) and thus, serum neutralization tests, which are sometimes used as gold standards for verifying ELISA results, cannot confidently be used to determine exposure to this virus. For this reason we decided to perform experimental transmission studies using red-ear sliders to attempt to demonstrate the validity of our assay by detecting seroconversion. Our assay failed to detect antibodies in all but one slider. However, recent reports show that multiple exposures are needed in amphibians to detect IgY antibodies against FV3 using an ELISA (Gantress *et al.*, 2003; Maniero *et al.*, 2006) and IgM antibodies were not detectable even after multiple exposures. Only one dose of virus was admininstered to experimentally inoculated turtles, which might explain why the turtles in this study failed to mount an immune response. Our results did show a good correlation between antibody production following a clinical disease in a Burmese star tortoise that was previously housed with a tortoise that died with confirmed *Ranavirus* infection. Plasma from a Burmese star tortoise from another facility with no known history of illness was selected as a seronegative control; antibodies to *Ranavirus* were not found in this tortoise.

Additionally, using a western blot, we were able to show that plasma from the positive control turtle bound to proteins in the infected cells in the immunoblot but not in the uninfected cells indicating that the plasma was reacting with viral proteins and not cellular proteins from the lysate we were using to coat the plates. For these reasons, we assume our test to be valid for use in detecting anti-iridovirus antibodies in chelonians, however further validation of this assay is warranted.

Only one turtle died as a result of IM inoculation of virus at 10^2TCID$_{50}$. A previous study showed that 75% (n=4) of turtles inoculated with a higher dose of virus (10^5TCID$_{50}$) died as a direct result of iridovirus infections (Johnson *et al.,* unpublished data). The viral dose was extrapolated from experimental studies with fish and amphibians (Langdon, 1989, Moody and Owens, 1994, Bollinger, 1999, Cullen and Owens, 2002), so it was unknown what a sub-lethal dose would be in chelonians. While studies with a larger sample size might provide more accurate correlations between viral load and mortality rates, it appears that host characteristics likely play a significant role in resistance or susceptibility to disease. Environmental temperatures have also been shown to significantly impact the percent mortality and time to death in salamanders experimentally inoculated with a *Ranavirus* (Rojas *et al.,* 2005), where salamanders inoculated at 18 and 10°C were more likely to die than those exposed at 26°C. Underlying disease conditions were not noted at necropsy or on histologic review of tissues in the turtle that died that might have contributed to an increased susceptibility.

Prevalence among free-ranging gopher tortoises was found to be low, only 1.5% of 1000 samples being positive. This could be the true prevalence rate, although we suspect that this is an underestimate of the true rate. There are several factors that could cause

the prevalence to be underestimated. Prevalence is a function of the incidence of disease multiplied by the average duration of the illness. While incidence would be difficult to determine in a natural setting, duration of illness can be extrapolated from experimental studies. If chelonians die quickly as a result of infection, they will not have time to mount an immune response to the pathogen, and will not survive to be surveyed. As previously mentioned, experimental transmission studies have shown a high rate of mortality (75%) in turtles intramuscularly inoculated with $10^5 TCID_{50}$ of a *Ranavirus* infected cell lysate (Johnson *et al.*, unpublished data). Turtles all died within 30 days of exposure to the virus. Although, the route of transmission is unknown in a natural environment, if naturally exposed cases experience similar mortality rates and duration from exposure to death, a cross-sectional study evaluating the prevalence of exposure will miss many tortoises that were exposed, because the majority of them will die. This was demonstrated in a natural setting in Pennsylvania. A population of approximately 70 eastern box turtles (*Terrapene carolina carolina)* was being repatriated in a nature sanctuary. Turtles were tracked every 3-5 days by radiotelemetry. In the summer and fall of 2003, 15 of these turtles died suddenly, with what was later idenitified to be iridovirus infections (Johnson *et al.*, 2004). The following spring, the remaining 55 turtles were sampled, and plasma was run for the presence of antibodies. Only three turtles were positive on ELISA (data not shown). If we calculated this value as the true prevalence, we would estimate that ~5.5% of turtles were exposed, when we know that 21% of the population died that was not included in the estimate. This severely underestimates the prevalence of disease. However, we cannot extrapolate these differences to other populations, as this was a repatriated population, and thus, subject to

stressors that might not be found in wild populations, making them more susceptible to disease. Secondly, this study suggests that turtles must be exposed more than once to mount an immune response similar to what was found in studies with *Xenopus* (Gantress *et al.,* 2003; Maniero *et al.*, 2006). Thus, if turtles have only been exposed once, we will not detect that first exposure with our ELISA, also underestimating the true prevalence of exposure.

Emerging infectious diseases have been increasingly recognized as factors influencing wildlife health and populations (Harvell *et al*., 1999; Daszak *et al*., 2000). Although mycoplasmosis has been postulated to contribute to declines of some tortoise species (USFWS, 1994), the cause(s) of mass mortality events in wild chelonian populations often remain undetermined (Flanagan, 2000; Dodd, 2001). Iridovirus infections in chelonians can have a high mortality rate, but the duration of illness is short, making it difficult to observe disease outbreaks in the wild. It is possible that it might contribute significantly to mortality rates in wild populations of chelonians. Utilizing a serological assay may help to determine regions where iridovirus infections might be more prevalent in chelonian populations. These locations could then be monitored more closely for disease outbreaks in both chelonians and amphibians.

Results of the serosurvey showed that counties in the central and southeastern region of Florida were more likely to have seropositive tortoises. Interestingly, four of these counties had adjoining borders (St. Lucie, Martin, Palm Beach and Broward), suggesting that either tortoises in this area are at higher risk of exposure to iridoviruses, or alternatively, iridovirus is more endemic in this area, so lower levels of exposure allow more tortoises to seroconvert without succumbing to the disease. Direct comparisons of

prevalence rates between counties are difficult to make as this was a convenience sample and geographic area or tortoise densities per county data were not controlled for in sampling. Only 32 of 67 counties were sampled. The northwestern region was not well sampled, and inferences about this region can't be made. However, seven tortoises in Baker County, Georgia were seropositive and this county is located in the southwestern portion of the state, which would be near the northwestern portion of Florida.

Earlier studies have shown that amphibians might be a source of iridovirus infections in chelonians (Johnson *et al.*, unpublished data). A moribund leopard frog was found and euthanized at the same site in Georgia where the Burmese star tortoises became ill and one died. Restriction enzyme analysis of viral genomic DNA from an isolate obtained from the tortoise and the frog demonstrated identical restriction patterns, suggesting they are the same or very closely related viruses. Thus, conditions that might propagate amphibian iridovirus infections would likely cause an increased chance of exposure in chelonians. Green *et al.*, (2002) found that increased precipitation and population densities were directly associated with increased die-offs of amphibians. Thus, these similar settings might create higher rates of exposure in chelonians. Additionally, it has been shown that sublethally infected amphibians can cause sporadic, recurrent disease outbreaks in amphibians (Brunner *et al*., 2004). Experimentally and naturally infected tiger salamander larvae and metamorphs were able to maintain sublethal, transmissible infections for over five months. Apparently healthy infected dispersing metamorphs were returning to water bodies to breed and it was speculated that these individuals were likely serving as a reservoir host for infecting newly hatched larvae, creating recurrent outbreaks of disease. It is unknown whether chelonians are

capable of sustaining sublethal infections or capable of spreading disease to naive populations. One turtle experimentally inoculated intramuscularly with iridovirus remained clinically healthy but continued to shed virus from the cloaca detectable by PCR up until 30 days. The study ended at 30 days so it is unknown how long this turtle would have kept shedding, or if the virus being shed was still infectious. Further studies would be useful to determine the risk posed to other chelonian and amphibian populations of iridovirus infected turtles that survive the initial infection. This assay could be useful for managing populations in wild and captive settings by identifying tortoises who might be asymptomatic carriers.

In summary, this study reports the development of an indirect ELISA for detection of anti-iridovirus antibodies in chelonians. It was able to detect antibodies in a naturally infected Burmese star tortoise whose pen-mate died with a confirmed iridovirus infection. A seroprevalence survey of banked plasma samples from free-ranging gopher tortoises in Florida, Georgia, Alabama, Louisiana and Mississippi found a 1.5% prevalence rate of exposure. Further studies are needed to characterize the true incidence of disease in wild populations of chelonians.

Table 3-1. Reproducibility of the ELISA. SD = standard deviation of the mean A_{405}. CV = coefficient of variance expressed as a percent. ICL = values from samples run on wells coated with infected cell lysate. UCL = values from samples run on wells coated with uninfected cell lysate.

		Positive Sample				Negative Sample			
		n	Mean A_{405}	SD	CV	n	Mean A_{405}	SD	CV
Intra-assay	ICL	64	0.363	0.022	6.06	64	0.107	0.011	10.28
	UCL	32	0.094	0.009	9.57	32	0.112	0.009	8.04
Inter-assay	ICL	26	0.366	0.079	21.58	26	0.130	0.023	17.69
	UCL	13	0.095	0.011	11.58	13	0.111	0.017	15.31

Table 3-2. ELISA results of 1000 free-ranging gopher tortoise (*Gopherus polyphemus*) plasma samples by county and state.

State	**County**	**Number tested**	**Positive**	**Percentage**
AL	Baldwin	2	0	0
AL	Mobile	7	0	0
FL	Alachua	12	0	0
FL	Brevard	27	0	0
FL	Broward	10	1	10.0
FL	Citrus	51	0	0
FL	Clay	1	0	0
FL	Collier	2	0	0
FL	Columbia	1	0	0
FL	Hernando	24	0	0
FL	Hillsborough	16	0	0
FL	Indian River	2	0	0
FL	Lake	99	3	3.0
FL	Lee	18	0	0
FL	Leon	17	0	0
FL	Madison	1	0	0
FL	Manatee	6	0	0
FL	Marion	22	0	0
FL	Martin	25	1	4.0
FL	Miami-Dade	8	0	0
FL	Nassau	47	0	0
FL	Orange	40	0	0
FL	Osceola	11	0	0
FL	Palm Beach	58	2	3.4

FL	Pasco	26	0	0
FL	Pinellas	2	0	0
FL	Polk	6	0	0
FL	Sarasota	1	0	0
FL	Seminole	54	0	0
FL	St. Johns	7	0	0
FL	St. Lucie	35	1	2.9
FL	Taylor	1	0	0
FL	Volusia	26	0	0
FL	Walton	2	0	0
GA	Baker	113	7	6.2
GA	Liberty	74	0	0
GA	Tattnall	38	0	0
LA	Washington Parish	12	0	0
MS	Greene	7	0	0
MS	Harrison	16	0	0
MS	Perry	5	0	0
	Unknown	68	0	0
TOTAL		**1000**	**15**	**1.5**

Table 3-3. ELISA results of 658 free-ranging gopher tortoises (*Gopherus polyphemus*) from the state of Florida are listed by region. C = central, CE = centraleast, CW = centralwest, NC= northcentral, NE = northeast, NW = northwest, SE = southeast and SW = southwest.

Region	Total No. Tested	No. Positive	Percent
C	232	3	1.3
CE	90	1	1.1
CW	126	0	0.0
NC	32	0	0.0
NE	55	0	0
NW	2	0	0
SE	101	4	4.0
SW	20	0	0

Table 3-4. ELISA results of 1000 free-ranging gopher tortoises (*Gopherus polyphemus*) listed by state.

State	Total No. Tested	Total No. Positve	Percent
Alabama	9	0	0
Florida	658	8	1.2
Georgia	225	7	3.1
Louisiana	12	0	0
Mississippi	28	0	0

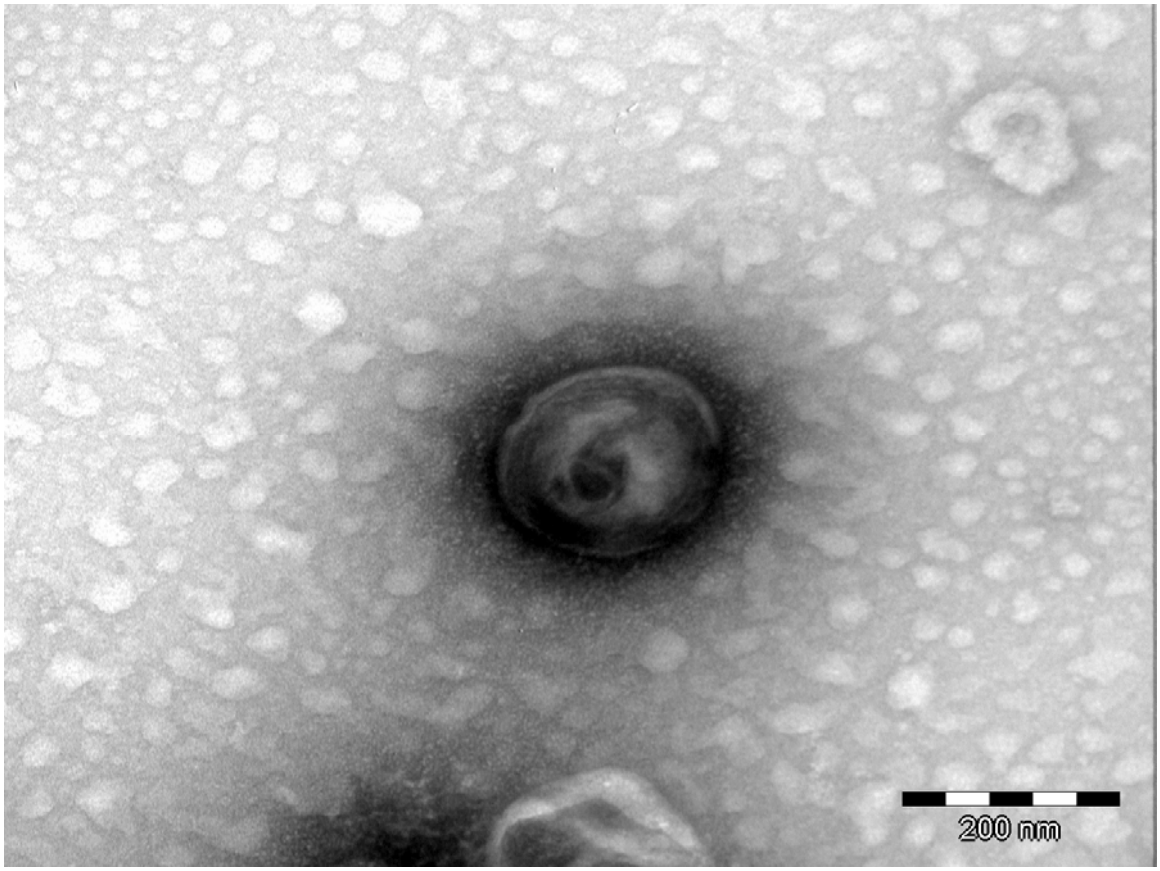


Fig. 3-1. Negative staining electron photomicrograph of an iridovirus particle purified by sucrose gradient ultracentrifugation.

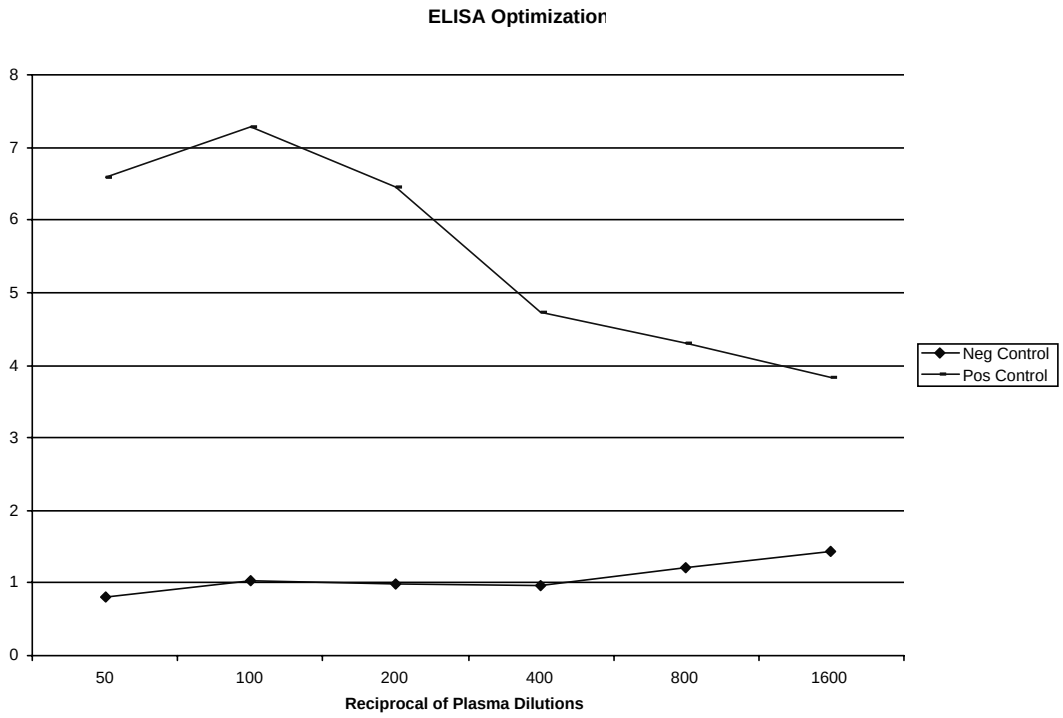


Fig. 3-2. Optimization of the ELISA with antigen coated at 1:100 dilution, comparing the positive to negative (P/N) ratio of two fold serial plasma dilutions of the positive control turtle (Burmese star tortoise with clinical signs of illness) versus a negative control (Burmese star tortoise with no history of illness). Plasma diluted at 1:100 showed the greatest difference between the positive and negative control.

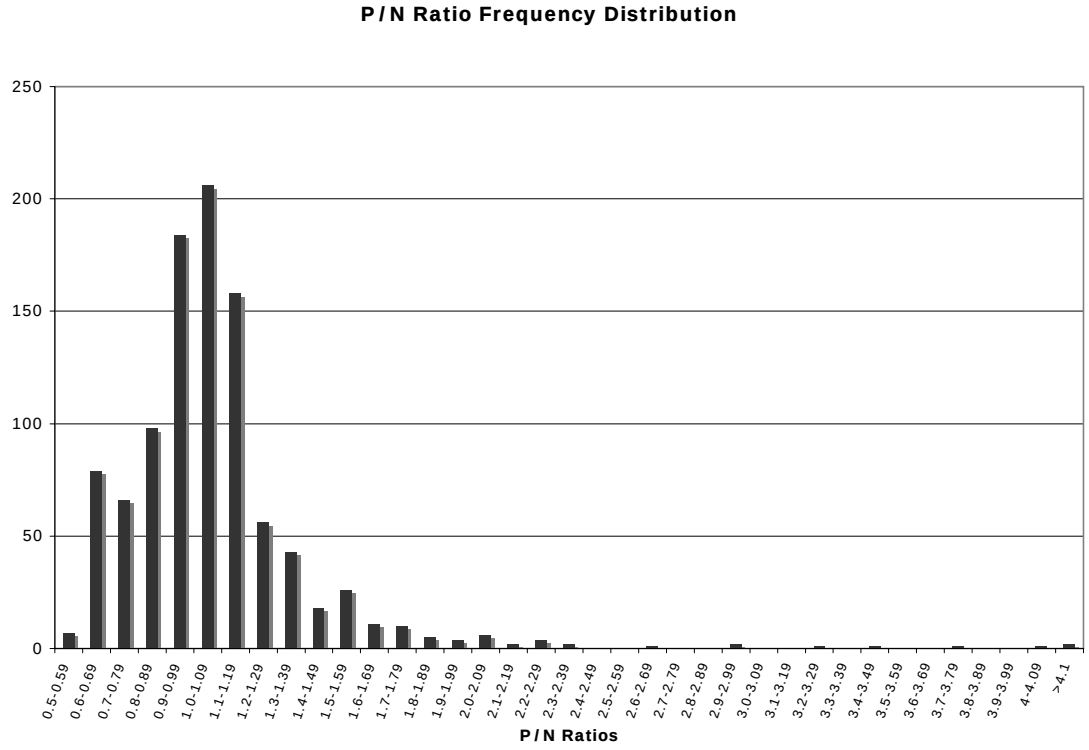


Fig. 3-3. Frequency distribution of P/N ratios from an indirect ELISA performed on 1000 free ranging gopher tortoise (*Gopherus polyphemus*) plasma samples. Samples show an approximately normal distribution, skewed to the right.

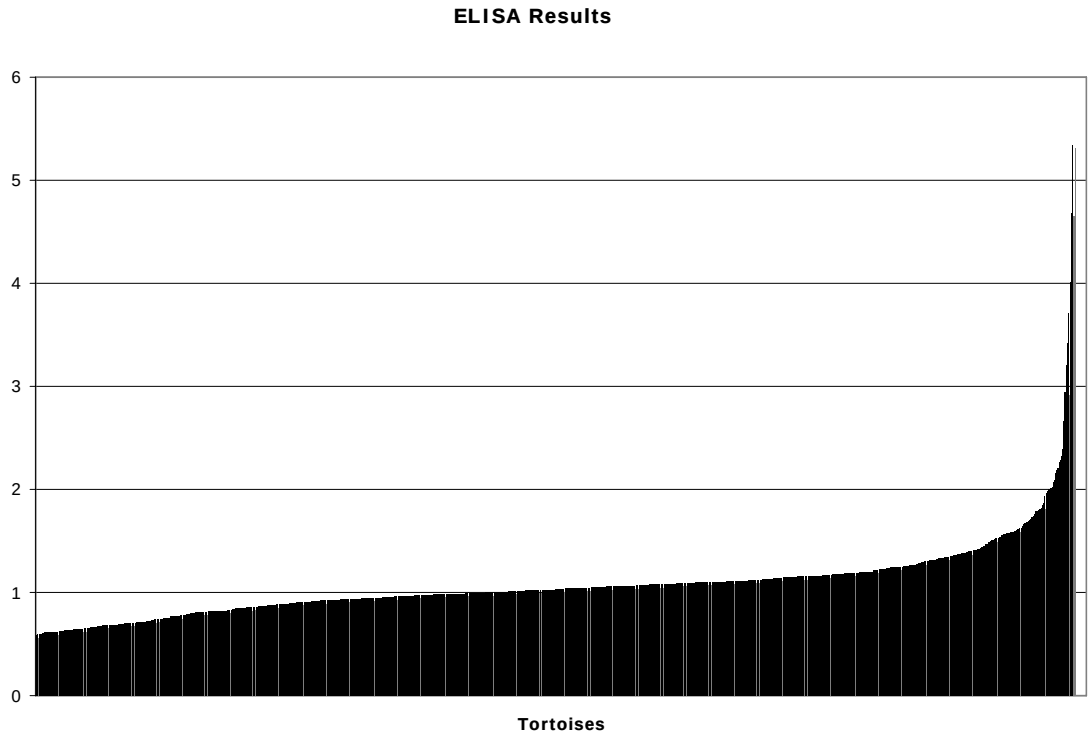
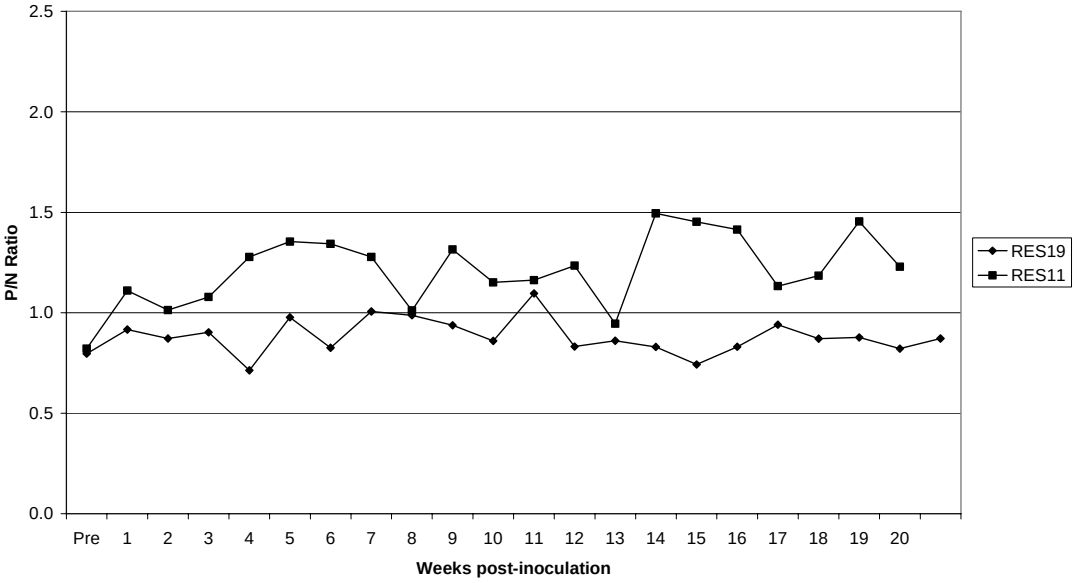
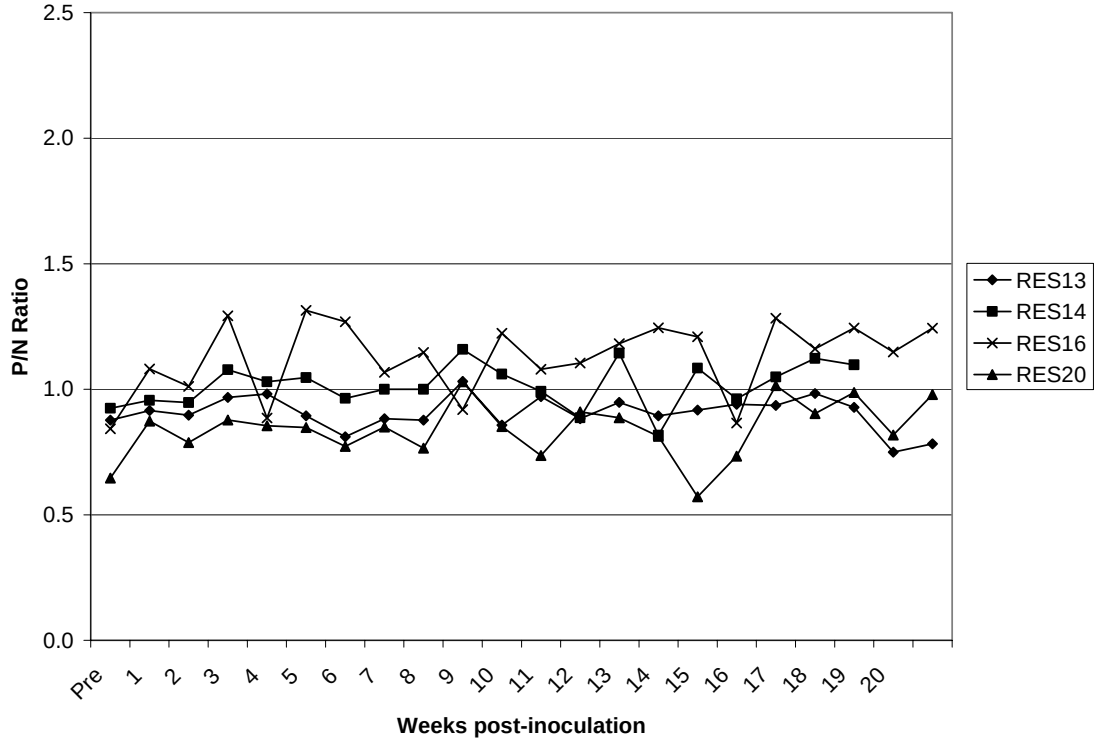


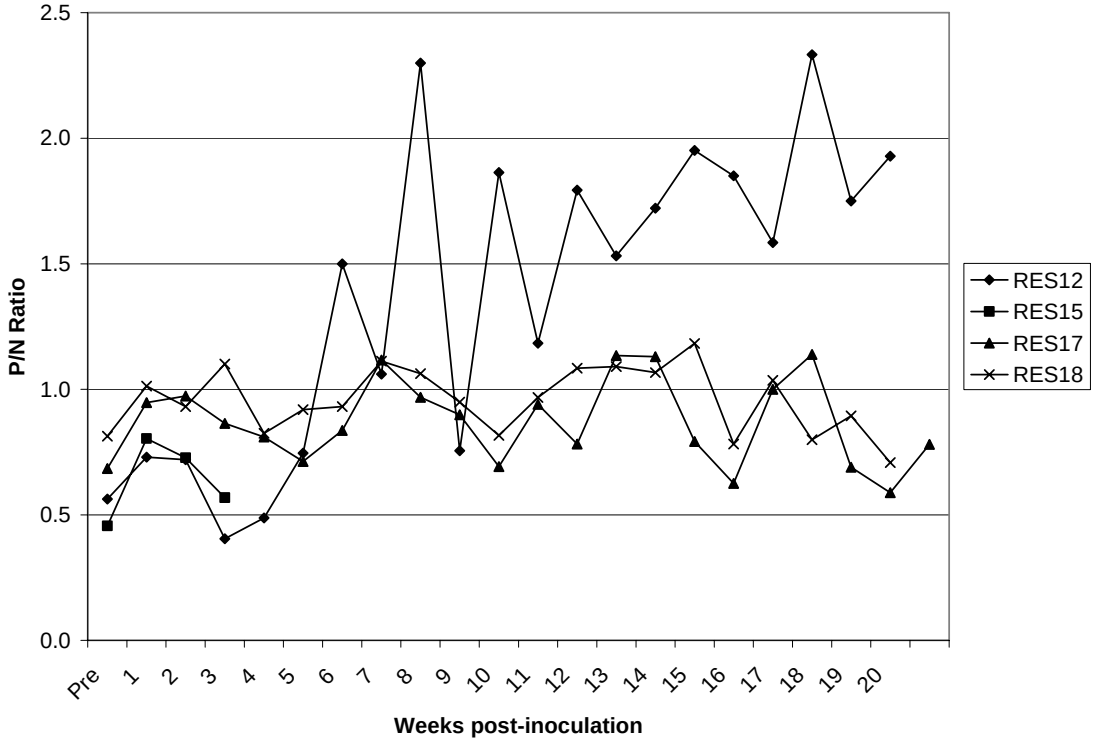
Fig. 3-4. Individual P/N ratio values for 1000 free-ranging gopher tortoises (*Gopherus polyphemus*) in increasing value.


2.5
2.0
1.5
1.0
0.5
0.0
P/N Ratio
Pre 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Weeks post-inoculation
RES19
RES11

A


2.5
2.0
1.5
1.0
0.5
0.0
P/N Ratio
Pre 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Weeks post-inoculation
RES13
RES14
RES16
RES20

B



C

Fig. 3-5. P/N ratios of red-eared slider (*Trachemys scripta elegans*) plasma samples collected weekly over five months. A) P/N ratios of mock-inoculated turtles. B) P/N ratios of turtles orally inoculated with *Ranavirus*. C) P/N ratios of turtles intramuscularly inoculated with *Ranavirus*.

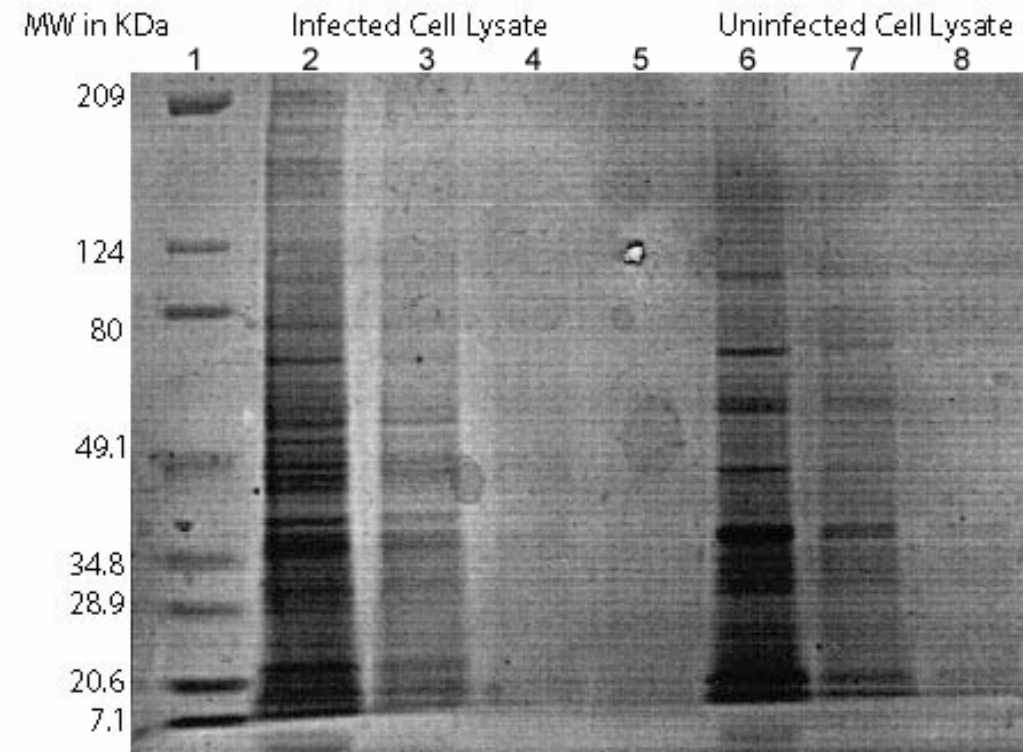


Fig. 3-6. Coomassie blue staining of a SDS-PAGE gel separating proteins of iridovirus-infected and uninfected *Terrapene* heart cell lysates. Lane 1 is a broad range, pre-stained molecular weight marker with weight in kDa marked next to the lane. Lanes 2-5 are four fold serial dilutions of iridovirus infected cell lysate. Lanes 6-8 are four fold serial dilutions of uninfected cell lysate.

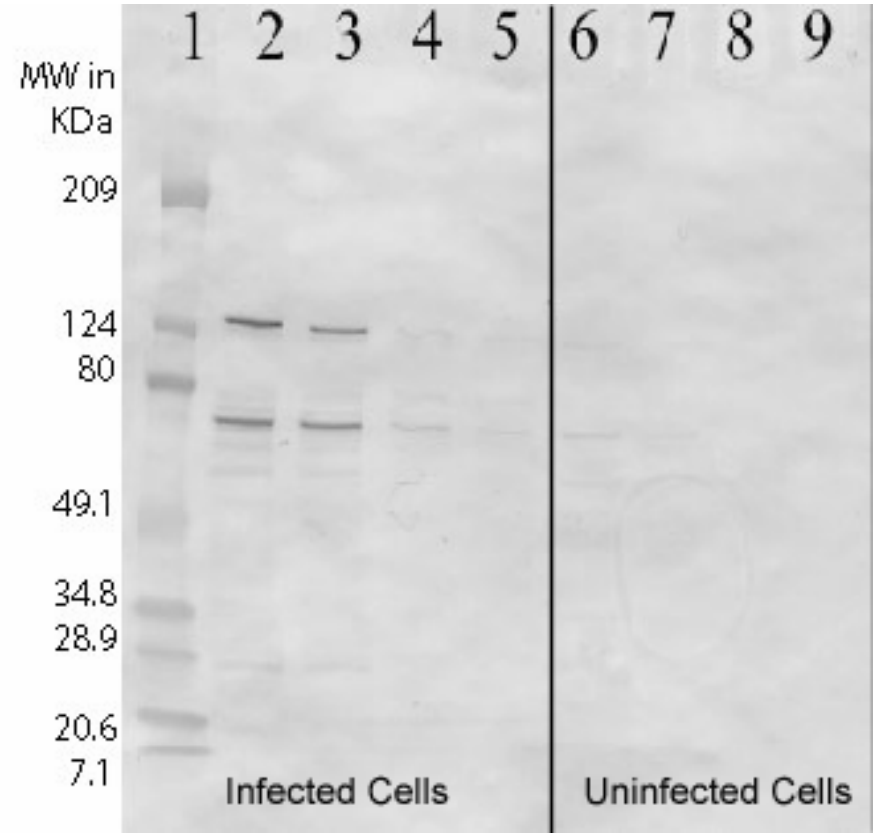


Fig. 3-7. Western immunoblot. Lanes coated with four fold serial dilutions of iridovirus infected cell lysate in lanes 2-5 and uninfected cell lysate in wells 6-9. Plasma from the positive control was used as the primary antibody for detection of iridovirus specific antibody binding. Strong signal was seen on virus-infected cells at approximately 125 kDa, and 78 kDa with weaker signals seen at 70, 65, and 28 kDa. Very faint signals were also seen at 125 and 78 kDa in the uninfected cells.

Fig. 3-8. County map of Florida. The five counties highlighted indicate where seropositive tortoise samples were identified.

CHAPTER 4
EXPERIMENTAL TRANSMISSION OF A *RANAVIRUS* IN WESTERN ORNATE BOX TURTLES (*Terrapene ornata ornata*) AND RED-EARED SLIDERS (*Trachemys scripta elegans*)

Introduction

Viruses in the family Iridoviridae are large double stranded DNA viruses capable of infecting ectothermic vertebrates, and invertebrates (Williams *et al*., 2005). Iridoviruses within the genus *Ranavirus* have been shown capable of infecting fish, amphibians and reptiles (Chinchar, 2002) and have emerged as major pathogens of free-ranging amphibians worldwide (Cunningham *et al*., 1996, Zupanovic *et al.,* 1998, Daszak *et al*., 1999). In a study of sixty-four amphibian morbidity and mortality events between 1996 and 2001, the most common cause of mortality events was infection with iridoviruses (Green *et al*., 2002).

Iridovirus infections in reptiles have been less well described than in amphibians and fish, with sporadic reports in chelonians, snakes, and lizards (Just *et al.,* 2001, Hyatt *et al*., 2002, Marschang *et al.,* 2005). Infection of a tortoise was first reported in 1982 in a spur-tailed Mediterranean land tortoise (*Testudo hermanni*) that had necrotic foci in the liver, intestine and spleen (Heldstab and Bestetti, 1982). Subsequently, iridoviruses were reported in other species of chelonians both in captivity and in the wild (Westhouse *et al.,* 1996, Chen *et al.,* 1999, Marschang *et al.,* 1999, DeVoe *et al.,* 2004). Clinical signs associated with infections have included signs of upper respiratory tract disease including respiratory distress and nasal discharge, as well as oral ulceration, cutaneous abscessation, anorexia and lethargy (Westhouse *et al.,* 1996, DeVoe *et al.,* 2004). While

there is circumstantial evidence that iridovirus is a primary pathogen in chelonians, Koch's postulates have never been fulfilled.

Ranavirus was recently identified in tissues obtained from a variety of wild and captive chelonians that died in Georgia, Florida, Texas, New York and Pennsylvania (Johnson *et al*., 2004). Species affected were Burmese star tortoises (*Geochelone platynota*), gopher tortoises (*Gopherus polyphemus*), eastern box turtles (*Terrapene carolina carolina*), and Florida box turtles (*Terrapene carolina bauri*). Clinical signs in these cases included lethargy, anorexia, nasal discharge, conjunctivitis, severe subcutaneous cervical edema, and necrotizing pharyngitis-stomatitis. One of the ranaviruses isolated from a Burmese star tortoise (*Geochelone platynota*) that died in an outdoor pen in a zoological collection from Georgia in 2003 was further characterized as either identical or closely related to Frog Virus 3 (Johnson *et al*., 2004). To determine a causal relationship between the isolated *Ranavirus* and the clinical and histologic lesions observed in these chelonians and to fulfill Koch's postulates, we performed two experimental transmission studies using the previously characterized Burmese star tortoise isolate that will hereafter be termed Burmese star tortoise *Ranavirus* (BSTRV). The first was a pilot study to determine the suitability of either western ornate box turtles (*Terrapene ornata ornata*) or red-eared sliders (*Trachemys scripta elegans*) as an experimental model for a subsequent larger transmission study. Here we report the findings of those studies.

Materials and Methods

Experimental Animals and Husbandry

Study 1 was a pilot study consisting of three western ornate box turtles (BT; *Terrapene ornata ornata*) and three red-eared sliders (RES; *Trachemys scripta elegans*)

that were purchased from a reptile supplier in Texas. Study 2 consisted of ten RES purchased from a reptile supplier in Louisiana. Both studies were performed under the approval of the Institutional Animal Care and Use Committee at the University of Florida. Turtles were housed individually in plastic containers in a centralized animal facility room maintained at approximately 25^{o}C. Appropriate husbandry for each species was used that included commercially available foods. Box turtles were kept on land, with overhead heat lamps provided for basking at one end of the container (average temperature of 28 ^{o}C). Red-eared sliders were kept in water (temperatures averaging between 21.3 and 25.6°C), with basking platforms provided under an overhead heat lamp (average basking temperature of 28°C). Fluorescent room lights were kept on a 12-hour light and 12-hour dark cycle.

Pre-inoculation Sample Collection

Upon arrival, each turtle was examined for the presence of any clinical signs of illness. Urine, oral and cloacal swabs were obtained from each turtle and tested for the presence of iridovirus utilizing a polymerase chain reaction (PCR) test (see below). Blood samples were collected from each turtle into 2ml tubes coated with lithium heparin and centrifuged at 4,500 x *g* for 5 min. Buffy coats were removed and also tested for iridovirus using PCR. Plasma was removed and tested for anti-*Ranavirus* antibodies using an indirect enzyme linked immunosorbent assay (ELISA; see below).

DNA Preparation, Polymerase Chain Reaction and Nucleotide Sequencing

Oral and cloacal swabs were combined for each turtle into one 1.5ml microcentrifuge tube and 100µl of phosphate buffered saline was added. DNA was extracted from buffy coats, oral and cloacal swabs using the DNeasy kit (Qiagen, Valencia, CA, USA) as were tissue samples later collected at necropsy. Viral DNA from

urine samples was extracted using the QIAmp Ultrasens virus kit (Qiagen). Sense primer (5'-GACTTGGCCACTTATCAC-3') and anti-sense primer (5'-GTCTCTGGAGAAGAAGAA-3'), were used as previously described (Mao *et al*., 1997) to amplify approximately 500 basepairs of the *Ranavirus* major capsid protein gene. A 20μl reaction mixture was run which contained 2μl extracted DNA, 1 μM of both primers, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1.5 U of *Taq* DNA polymerase and PCR buffer containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ (Eppendorf, Westbury, New York). The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid) with an initial denaturation at 94˚C for 2.5 min, followed by 25 cycles of denaturation at 94˚C for 30 sec.; annealing at 50˚C for 30 sec, extension at 72˚C for 30 sec., and a final extension step at 72˚C for 10 min as previously described (Marschang *et al*., 1999). The PCR products were resolved in 1% agarose gels and any bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Products were sequenced in both directions directly using the Big-Dye Terminator Kit (Perkin-Elmer, Branchburg, New Jersey) and analyzed on ABI 377 automated DNA sequencers at the University of Florida's Sequencing Center. The sequences were compared to known sequences in GenBank (National Center for Biotechnology Information, Bethesda, Maryland), EMBL (Cambridge, United Kingdom), and Data Bank of Japan (Mishima, Shiuoka, Japan) databases using TBLASTX (Altschul *et al*., 1997).

ELISA

An indirect enzyme linked immunosorbent assay (ELISA) was used to determine the presence of anti-*Ranavirus* antibodies. The ELISA methodology was similar to that developed for use in identifying the presence of anti-tortoise herpesvirus antibodies in

tortoises (Origgi *et al*., 2004) and anti-west Nile virus antibodies in alligators (Jacobson *et al*., 2005). The BSTRV isolate was used as the antigen in the assay. Each well of a 96 well high-protein binding microplate was coated overnight at 4 ^{o}C with 50 µl of a 1:400 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 BSTRV. Lysates were diluted in 0.01 M sodium phosphate buffer (pH7.2) containing 0.15 NaCl and 0.02% NaN_3 (PBS/A). Wells were then washed four times in ELISA wash buffer (PBS/A 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 were incubated for one hour at room temperature. Plasma samples were added in 50µl volumes at a 1:100 dilution in blocking buffer. The secondary antibody used was a biotin-conjugated mouse anti-tortoise immunoglobulin (Ig) monoclonal antibody diluted to a final concentration of 0.5µg/ml in blocking buffer. Alkaline phosphatase-conjugated-streptavidin (Zymed Laboratories, Inc., San Francisco, CA) was then applied to each well at 50 µL of a 1:5000 dilution in PBS/A. Next, 100 µl of a 1.0 mg/ml P-nitrophenyl phosphate prepared in 0.01 M sodium bicarbonate buffer containing 2 mM $MgCl_2$ was added to each well and the plates were then stored in the dark until being read. The optical density (OD) of each well was read at A_{405} using a StatFax 3200 microplate reader (Awareness Technology, Palm City, Florida, USA) after 30 minutes.

Each sample was done in triplicate: one time on wells coated with uninfected cell lysate and in duplicate on wells initially coated with infected cell lysate. The replicate values of the wells coated with BSTRV lysate were averaged and divided by the OD

reading of the value of the plasma sample run on the uninfected cell lysate to subtract out any background binding that might be caused by cross-reactivity to the cells. Values greater than 2 were considered positive (Jacobson *et al*., 2005), suggesting previous exposure to the virus, and would preclude use of that turtle in the study. Plasma from a surviving penmate of the above Burmese star tortoise was used as a positive control.

Virus Preparation

Terrapene heart cells (TH-1) were acquired from the American Type Culture Collection (ATCC-CCL 50; Rockville, MD) and grown to confluency in 225cm^2 tissue flasks (Costar, Corning, NY). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60mg/liter; Sigma, St. Louis, MO), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300µg/liter; Sigma). Cells were inoculated with a fourth passage of BSTRV and incubated at 28ºC in the presence of 5% CO_2. When cytopathic effect (CPE) was observed, consisting of cell rounding and detachment from the flask in over 70% of cells, the flasks were scraped and contents transferred to 15 ml centrifuge tubes and clarified by slow speed centrifugation at 4,500x*g* for 30 minutes. The supernatant was then discarded and the cell pellet resuspended in 10mls of cell culture media. The preparation was then vortexed, frozen and thawed three times to release virus from the cells into the supernatant. The preparation was again clarified by centrifuging for thirty minutes. The supernatant was then transferred to a new tube and the cell pellet was discarded. The live virus in the media was then quantified by a $TCID_{50}$ assay, diluted with media to create a concentration of $10^5 TCID_{50}$/ml, and frozen at -80°C until use.

Transmission Studies

Study 1

Study 1 was designed as a pilot study to determine the suitability of either red-eared sliders (RES) or western ornate box turtles (BT) as a model of *Ranavirus* infection for chelonians. Three RES and three BT were included in the study and each turtle was allowed to acclimate for two weeks prior to infection. One of each species was assigned to one of three groups: 1) a mock inoculated control group, with both turtles receiving 0.5ml uninfected cell lysate orally and 0.5ml by intramuscular injection, 2) an orally (PO) inoculated group with both turtles receiving 1ml of infected cell lysate containing virus at $10^5 TCID_{50}$/ml by metal gavage feeding tube in the caudal esophagus and 3) an intramuscularly (IM) inoculated group, with both turtles receiving the same concentration of virus as the PO inoculated group with 0.5ml injected into both the left and right pectoral muscles. Turtles were observed daily after inoculation for the duration of the study. Oral swabs and buffy coats were collected 1-week post inoculation for evaluation by PCR for the presence of iridovirus. DNA extraction and PCR were performed as described previously for prescreening. Physical examinations were performed daily to assess the presence of the following clinical signs: lethargy, anorexia, cervical edema, palpebral or periocular edema, ocular discharge, nasal discharge, oral discharge, the presence of oral plaques or any other abnormalities.

Turtles were euthanized if clinical signs of disease became severe or at two weeks post-inoculation. For euthanasia, ketamine was administered intramuscularly at 100 mg/kg followed by intravenous sodium pentobarbital. Once turtles were unresponsive to painful stimuli and showed no corneal reflex, they were decapitated and a complete necropsy performed. Portions of tongue, esophagus, stomach, small and large intestine,

liver, kidney, and spleen were collected aseptically by flame sterilizing tools between each organ, and frozen at -80°C for virus isolation and/or DNA extraction and PCR. The following tissues were collected and fixed in 10% neutral phosphate buffered formalin: tongue, esophagus, stomach, small and large intestine, liver, kidney, spleen, pancreas, heart, trachea, lung, brain, urinary bladder, thyroid gland, adrenal gland, bone, bone marrow, skin, skeletal muscle, nasal cavity, eye and gonad. These tissues were then processed for histologic examination. They were embedded into paraffin, and 6 μm sections were stained with hematoxylin and eosin.

Virus isolation was performed on *Terrapene* heart cells seeded into 25 cm^2 flasks (Costar, Corning, NY). Cells were cultured in Dulbecco's modified Eagle medium/F12 (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco, Carlsbad, CA, USA), gentamicin (60 mg/liter) (Sigma, St. Louis, MO, USA), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300μg/liter) (Sigma) and cultured to confluency. A small piece of kidney (approximately 50mg) collected aseptically at necropsy was homogenized in separate 5ml tissue grinders containing DMEM. The homogenate was passed through a 0.45μm filter (Costar) onto a flask of cells. Cells were incubated at 28ºC and observed daily. Flasks of cells were harvested when CPE was observed in over 70% of cells or at post-inoculation day 10. DNA was extracted from cells using the DNeasy kit (Qiagen) protocol for animal cells. Polymerase chain reaction and nucleotide sequencing was performed as previously described to confirm the presence of iridovirus DNA sequences.

Study 2

Based on the results of the pilot study, a second transmission study was designed that consisted of ten RES. Turtles were assigned to one of the following three groups: 1) control; 2) IM inoculated or 3) PO inoculated. Turtles were numbered from one to ten according to increasing weight. They were then blocked into two groups (1-5 and 6-10) and turtles within each block were randomized to 1 control, 2 PO inoculated (turtle Nos. 1, 2, 7 and 10) and 2 IM inoculated (turtle Nos. 3, 5, 6 and 8). The control turtle in the lower weight block (turtle No. 4) was PO mock inoculated with 1ml of uninfected cell lysate, while the control turtle in the higher weight block (turtle No. 9) received 1ml IM, half in each pectoral muscle. Turtles were monitored daily for the presence of clinical signs consistent with iridovirus infection. Oral and cloacal swabs were taken three times a week throughout the four-week study. Free catch urine samples were collected opportunistically at the same time periods. Turtles were euthanized when clinical signs of infection became severe or four weeks post-inoculation.

Necropsies, tissue collections and virus isolation were performed as described in Study 1. In addition, spleen from one IM inoculated (No. 6) and one control (No. 9) turtle were collected in Trump's solution (4% formaldehyde, 1% glutaraldehyde in a phosphate buffer; Electron Microscopy Sciences, Hatfield, PA) for transmission electron microscopy (TEM). Tissues were embedded in Spurr’s resin and ultrathin sections were obtained and stained with lead citrate and uranyl acetate for TEM at the Electron Microscopy Core Laboratory, University of Florida.

Results

Experimental Animals and Pre-inoculation Sampling

Upon arrival, all turtles were considered healthy. Using PCR, all oral swabs, cloacal swabs, urine and buffy coats collected from turtles in Studies 1 and 2 were negative for iridovirus DNA sequences. All plasma samples evaluated by ELISA were below the positive cutoff value, indicating that the turtles were seronegative for exposure to *Ranavirus*.

Transmission Studies

Study 1

Control mock inoculated and PO inoculated red-eared sliders and box turtles showed no clinical signs of disease throughout the two-week study. The IM inoculated RES and BT both showed severe clinical signs. The RES died 9 days post inoculation (DPI). At 8 DPI the IM inoculated RES was basking continuously and started showing signs of lethargy, cutaneous erythema, and kept its palpebrae closed. It was found dead on the basking platform on the morning of 9 DPI. The orally inoculated RES remained normal. At 8 DPI, the IM inoculated BT developed a white opaque ocular discharge (Fig. 4-1), at ten days became lethargic and anorexic, and at 12 DPI, was euthanized. The orally inoculated BT remained normal.

Both IM inoculated turtles in the pilot study were positive by PCR for iridovirus sequences using DNA extracted from oral swabs and buffy coats collected one week post-inoculation. Oral swabs and buffy coats collected on the PO inoculated turtles and mock-inoculated control turtles were negative. PCR performed on DNA extracted from both IM inoculated turtles were positive for all eight tissues (Table 1). PCR was negative for all eight tissues of both control turtles and both PO inoculated turtles.

The most consistent and significant histologic lesions were observed only in the spleens (2/2) of IM inoculated BT and RES, both having similar changes. Changes were based on the normal histology of the spleen that was previously defined for red-eared sliders (Kroese and Van Rooijen, 1982). Lesions were centered on the splenic ellipsoids or "sheathed capillaries" and will be described in detail for turtles in Study 2 (below). Briefly, the walls of the ellipsoids were moderately to markedly expanded by homogenous to slightly fibrillar eosinophilic material (fibrin; fibrinoid vasculitis) with multifocal infiltrates of low numbers of heterophils. There were occasional lumenal fibrin thrombi with admixed heterophils and karyorrhectic debris.

Kidney samples from both IM inoculated turtles (P3 and P6) that were coated onto TH-1 cells demonstrated cytopathic effects of cell rounding and lysis within two days of incubation. Intracytoplasmic inclusion bodies were observed in infected cells and PCR and nucleotide sequencing on DNA extracted from cells from each flask were positive for iridovirus. None of the cultures inoculated with tissues collected from PO inoculated turtles, or control turtles demonstrated any CPE up to ten days post-inoculation of cells and each were negative by PCR for the presence of *Ranavirus* DNA segments.

Study 2

Similar to Study 1, only IM inoculated RES showed clinical signs of disease and were euthanized before the end of the four-week study. Three of the four IM inoculated turtles showing severe clinical signs were euthanized on days 11, 13 and 23 DPI. All three turtles became anorectic, and extremely lethargic. The turtle euthanized 13 DPI developed oral plaques on the roof of the mouth and tip of the tongue (Fig. 4-2). Turtle 3, euthanized 23 DPI, exhibited exophthalmus, conjunctivitis and hyphema (Fig. 4-3). All three turtles had clear serous ocular and nasal discharge. The fourth IM inoculated

turtle (No. 8) showed ocular discharge and subjectively basked more than the other turtles between 16 and 25 DPI, but then recovered and showed no clinical signs at the termination of the study. It became anorectic 14 DPI and remained so throughout the study. Three of 4 PO inoculated turtles also became anorectic after inoculation and remained so throughout the study. No other signs of disease were noted in those turtles.

Turtles mock inoculated were negative by PCR on all oral and cloacal swabs collected (Table 2). Three turtles PO inoculated (Nos. 2, 7 and 10) were positive by PCR on oral and/or cloacal swabs 2 DPI but not in any subsequent samples. The fourth PO inoculated turtle (No. 1) was not positive on any sample date. Oral and cloacal swabs from IM inoculated turtles were positive by PCR for iridovirus at varying times throughout the study (Table 2). Turtle No. 8 (IM inoculated), which was euthanized at the end of the study, was never positive on any oral swabs collected, but was positive on cloacal swab on five occasions including the last two sampling dates that were 23 and 26 DPI. PCR on urine samples followed a similar pattern. Three of four IM inoculated turtles were positive between one and five days prior to being euthanized (Table 3). One positive band from each turtle was sequenced to confirm the positive PCR results. All amplicons were of the expected size and each sequence shared 100% identity with the sequence of the original isolate. DNA extracted from eight tissues from each turtle including tongue, esophagus, stomach, small intestine, large intestine, kidney, spleen and liver were positive by PCR on three of four IM inoculated turtles (Nos. 3, 5 and 6) at necropsy (Table 1). Tissues from IM inoculated turtle No. 8, and all PO inoculated turtles and control mock-inoculated turtles were negative.

At necropsy, gross changes were observed in several turtles. Turtle No. 3 that was IM inoculated exhibited petechia in several organs including the glottis, liver, pancreas and fat. Congestion was observed in the stomach and on the surface of the bladder. The cecum and colon demonstrated a multifocal to coalescing area of hemorrhage (Fig. 4-4). The gastrointestinal tract of turtles 3, 5 and 6 appeared thickened and edematous. Turtle 6 also exhibited petechia on the surface of the pancreas and congestion in the stomach. No lesions were seen in PO inoculated and control group turtles.

Significant histologic lesions were observed only in IM inoculated turtles. Similar to the pilot study, the most consistent lesions were in the spleen (3/4 turtles) and centered on the splenic ellipsoids. The majority of the splenic white pulp surrounded ellipsoids (periellipsoidal lymphocyte sheath; PELS) (Fig. 4-5A and 4-6A) with lesser white pulp surrounding arterioles (periarteriolar lymphocyte sheath; PALS) (Fig. 4-6B). Ellipsoids (Fig. 4-6A) were characterized by plump, cuboidal, endothelial cells, a thick eosinophilic wall lacking smooth muscle (confirmed with Masson's trichrome stain), and a lack of reticular fibers between lymphocytes of the PELS using Gordon and Sweet's reticulin stain. In IM inoculated turtles, the walls of the ellipsoids were moderately to markedly expanded by homogenous to slightly fibrillar eosinophilic material (fibrin; fibrinoid vasculitis) (Fig. 4-5B and 4-6B) with multifocal infiltrates of low numbers of heterophils and scattered free brown pigment granules (presumptively from disrupted melanomacrophage centers). There were occasional lumenal fibrin thrombi with admixed heterophils and karyorrhectic debris (Fig. 4-6B). There was mild to moderate lymphoid depletion and dispersion of lymphoid cells in the PELS with relative sparing of

lymphocytes in the PALS (Fig. 4-5B and 4-6B). Replacing the PELS were combinations of karyorrhectic debris, heterophilic infiltrates, extravasated erythrocytes and fibrin.

The liver of all turtles had mild to marked hepatocellular vacuolar change that was most pronounced in the control turtles (Fig. 4-7A) and considerably less prominent in IM inoculated turtles (depletion of hepatocellular lipid and glycogen) (Fig.4-7B). Three (No. 3, 5, 6) of four turtles had multifocal random dilatation of hepatic sinusoids with fibrin thrombi (Fig. 4-7B) and variable single-cell necrosis of adjacent hepatocytes (Fig. 4-7C). Rarely, hepatocytes had small intracytoplasmic basophilic inclusion bodies (Fig. 4-7C). Admixed with fibrin thrombi and necrotic hepatocytes were small amounts of karyorrhectic debris, infiltrates of small numbers of heterophils, and for lesions occurring adjacent to melanomacrophage centers, small amounts of dispersed brown granular pigment. One turtle (No. 3) had moderate multifocal hemorrhage in association with liver lesions as well as moderate multifocal fibrin thrombi within small to medium portal venules and veins.

In addition to vascular changes in splenic ellipsoids, hepatic sinusoids and portal blood vessels, acute fibrin thrombi were also observed in a variety of other tissues in intramuscularly inoculated animals (Fig. 4-8 and 4-9). Thrombi were noted in gastric or intestinal lamina propria, submucosa and serosa (No. 3, 5, and 6), glomerular capillaries (No. 3 and 6), esophagus (No. 3), pulmonary capillaries and veins (No. 3 and 6), meninges (No. 3 and 6), eye (No.3), nasal mucosa (No. 3), and oral mucosa (No. 3). Lesions associated with thrombi included segmental marked colonic mucosal hemorrhage (Fig. 4-9), multifocal mild to moderate meningeal hemorrhage, mild to moderate heterophilic meningitis, and mild heterophilic interstitial pneumonia.

A small number of IM inoculated turtles also had multifocal mild to moderate ulcerative and heterophilic stomatitis and esophagitis (No. 6) (Fig. 4-10), and multifocal mild to moderate heterophilic and necrotizing gastritis (No. 5 and No. 6). Turtles from all three groups had small granulomas in a wide variety of tissues that surrounded probable spirorchid-type trematode eggs and rarely, adult trematodes were observed within mesenteric blood vessels. Effete granulomas in the gastric submucosa and serosa of a small number of control and virus inoculated turtles contained cross-sections of an unidentified nematode. Trematodes and nematodes were interpreted as incidental findings.

Similar to study 1, cytopathic effects consisting of cell rounding and lysis were seen in cultures of *Terrapene* heart cells that were coated with kidney homogenates from three of four IM inoculated turtles (Nos. 3, 5 and 6). Using PCR and nucleotide sequencing, *Ranavirus* was identified in DNA extracted from cells from each flask. No CPE was seen in cells that received tissue homogenates from orally inoculated turtles, control turtles, and one of the intramuscularly inoculated turtles (No. 8).

Transmission electron microscopy of splenic ellipsoids in 1 IM inoculated turtle (No. 6) in the full transmission study demonstrated marked expansion of the vessel wall by a granular to fibrillar lightly electron dense material that was consistent with fibrin. Admixed with fibrin and within remnants of the white pulp were scattered unidentified necrotic cells with intracytoplasmic arrays of icosahedral virions consistent with an iridovirus. No virions were observed in cells associated with ellipsoids in the control turtle (No. 9).

Discussion

Henle-Koch's postulates provide a strict set of guidelines for proving a causal relationship between an infectious agent and a disease (Evans, 1976). Here we performed a transmission study using a *Ranavirus* isolate from a captive Burmese star tortoise that became ill and died in an attempt to determine if a causal relationship exists between infection with this *Ranavirus* and the clinical and histologic changes observed in the Burmese star tortoise. Since it is not practical to perform a challenge study in this critically endangered species, we decided to assess both box turtles and red-eared sliders as a model for *Ranavirus* infection in chelonians. Box turtles were selected since *Ranavirus* infection has been identified in this species (Mao *et al*., 1997; Devoe *et al*., 2004; Johnson *et al*., 2004). We decided to also evaluate the suitability of red-eared sliders since populations of box turtles are declining throughout their range. Red-eared sliders, however, are being raised in the lower Mississippi Valley for the overseas pet trade, which became a factor in their ultimate selection as an experimental animal in our studies. In addition, results of the pilot study showed that both species similarly responded when administered a *Ranavirus* isolate by two different routes. Both IM inoculated turtles showed severe clinical signs and were euthanized prior to the termination of the study at two weeks. Oral swabs from both were positive one week post-inoculation and histologic lesions were consistent between the two species. Both the PO inoculated RES and BT did not show any clinical or histologic lesions. Results of this study showed that both RES and BT can serve as suitable models of *Ranavirus* infection for chelonians. As a result, RES were chosen as the experimental model for the larger transmission study.

Histologic lesions in turtles inoculated by intramuscular injection in both studies consisted primarily of multicentric fibrinoid vasculitis and formation of fibrin thrombi in small blood vessels in numerous tissues, and in this regard, resembled recent reports of *Ranavirus* infection in captive and free-ranging box turtles and tortoises (DeVoe *et al*., 2004; Johnson *et al*., 2004). Lesions in blood vessels were consistent with observed *Ranavirus* infection of endothelial cells in a naturally-infected captive Burmese star tortoise (Johnson *et al*., 2004) and with descriptions of apparent viral endotheliotropism in rainbow trout and redfin perch infected with another *Ranavirus* called epizootic haematopoietic necrosis virus (EHNV) (Reddacliff and Whittington, 1996). Involvement of endothelial cells may also be part of the pathogenesis of some amphibian *Ranavirus* infections as suggested by multicentric hemorrhage and edema or observation of characteristic inclusion bodies within endothelial cells (Wolf *et al*., 1968; Cunningham *et al*., 1996; Bollinger *et al*., 1999; Docherty *et al*., 2003).

The consistent involvement of the splenic ellipsoids (sheathed capillaries) provides a basis for the prominent necrotizing splenitis observed in some natural *Ranavirus* infections of chelonians (DeVoe *et al*., 2004; Johnson *et al*., 2004). Similar lesions of the ellipsoids were observed in redfin perch, but not rainbow trout, experimentally infected with EHNV (Reddacliff and Whittington, 1996). A filtering function for splenic ellipsoids for particulate material and immune complexes has been documented in other species (Sorby *et al*., 2005) and possibly, the lesions observed in chelonian *Ranavirus* infections could be a consequence of antigen trapping in macrophages associated with the ellipsoid sheath during ranaviral viremia.

Necrotizing stomatitis, esophagitis and/or pharyngitis are characteristic lesions in many naturally occurring chelonian *Ranavirus* infections (Westhouse *et al*., 1996; Marshang *et al*., 1999; DeVoe *et al*., 2004; Johnson *et al*., 2004). Clinical signs such as ocular and nasal discharge, conjunctivitis and palpebral edema associated with *Ranavirus* infection (both naturally infected animals and the experimental animals in this report) are often attributed to the upper respiratory tract and overlap with those signs observed with mycoplasmosis caused by *Mycoplasma agassizii* (Brown *et al*., 1999) or herpesvirus infection (Origgi, *et al*., 2004; Johnson *et al*., 2005). In particular, infection with tortoise herpesviruses 1 and 2 are associated with caseous or diphtheritic oral plaques that are grossly indistinguishable from oral lesions associated with *Ranavirus* infection. In the experimentally infected animals of this report, necrotizing stomatitis and esophagitis were observed in a single intramuscularly inoculated red-ear slider (No. 6). Lesions were not observed in the nasal cavity of any animal examined. Oral lesions could be secondary to thrombus formation and infarction in small submucosal vessels or alternatively, could be the result of viral infection and necrosis of oral epithelial cells. The observation of intracytoplasmic inclusion bodies consisting of *Ranavirus* in epithelial cells of some naturally occurring infections would appear to support the latter explanation. Why oral lesions were not present in more of the experimentally inoculated turtles in this report is uncertain, but it is possible that epithelial infection is a late event that follows viremia and hence was not observed in experimental animals. Also, the virus may have been attenuated in cell culture, resulting in altered pathogenicity.

The intracytoplasmic basophilic inclusion bodies that are suggestive of iridovirus infection and prominent in many cases of *Ranavirus* infection in fish, amphibians and

some chelonians were not prominent in the experimentally inoculated turtles (Reddacliff and Whittington, 1996; Westhouse *et al*., 1996; Bollinger *et al*., 1999; Marschang *et al*., 1999; Docherty *et al*., 2003). This observation is consistent with recently reported naturally occurring chelonian *Ranavirus* infections, and indicates that inclusion bodies may be an inconsistent finding and should not be relied upon for use in formulating a histologic differential diagnosis (Devoe *et al*., 2004; Johnson *et al*., unpublished findings). Virions consistent with ranaviruses were observed by transmission electron microscopy in cells within the spleen of an IM inoculated red ear slider and suggests that TEM may still be a useful diagnostic tool in chelonian *Ranavirus* infections even in the absence of visible inclusions on histologic section. Demonstration of intracytoplasmic virions in cells of an experimentally inoculated turtle is important because it shows that the virus is capable of entering and replicating within cells, and that, lesions were not induced by the presence of inoculated non-replicating virus. Necrotizing liver lesions have been experimentally induced in mice and rats following injection of inactivated iridovirus virions or solubilized structural proteins (Lorbacher de Ruiz, 1990). Similarly, the *Ranavirus* Frog Virus 3 can trigger apoptosis in tissue culture in the absence of viral gene expression (Chinchar, 2002). Future studies may better define the *in-vivo* mechanism of cell death associated with iridovirus infections.

This study found that IM inoculated turtles were more likely to become infected with *Ranavirus*, show clinical signs and die compared to turtles that were orally inoculated. All four IM inoculated turtles showed clinical signs and three died as a result of infection (75%), whereas no orally inoculated turtles showed any signs of disease or died. This could mean that turtles do not become exposed through ingestion of infected

animals or water sources as has been previously shown to occur in amphibians (Jancovich *et al*., 2001, Pearman *et al.*, 2004), or that abrasions naturally acquired from ingesting bones or other abrasive material may be necessary for virus to be introduced systemically. Another explanation for the inability to re-create disease in orally inoculated animals was that a natural exposure was not replicated in the laboratory setting. Viral dose administered was extrapolated from studies done with fish and amphibians (Langdon, 1989, Moody and Owens, 1994, Bollinger, 1999, Cullen and Owens, 2002) but requirements for infection of turtles may be higher or repeated exposure may be necessary. Experimental infections of salamanders with a *Ranavirus* showed that both dose and host characteristics influenced the virulence of infection (Brunner *et al*., 2005). The infection dose was positively correlated with the mortality rate and inversely related to average survival times. Environmental temperatures have also been shown to significantly impact the percent mortality and time to death in salamanders experimentally inoculated with a *Ranavirus* (Rojas *et al*., 2005), where salamanders inoculated at 18 and 10°C were more likely to die than those exposed at 26°C. While water and room temperatures averaged between approximately 21 and 25°C, basking areas were kept warmer at 28°C. Eliminating heat lamps over basking areas and lowering the room temperature might have kept turtles cooler, and altered the results in the orally inoculated group. Alternatively, other routes of transmission such as vector-borne transmission may be required for turtles to become infected in the wild. Intracytoplasmic inclusion bodies were recently identified in the circulating leukocytes of an eastern box turtle infected with iridovirus (Allender *et al*., In Press). Ranaviruses are variably host specific, so virus may be able to survive in mosquitoes or other biting

insects capable of transmitting virus from an infected animal to an uninfected one. This has been shown to be the case with insect iridoviruses, where parasites were experimentally shown capable of transmitting invertebrate iridescent virus from infected to uninfected larvae (Lopez *et al*., 2002). It remains unknown whether natural outbreaks of iridovirus infections in any vertebrate species occur as a result of introduction of novel virus strains, recrudescence of latent or persistent infections in surviving populations as a result of stressors or other immunosuppressive causes, or viral persistence in the environment (Williams *et al*., 2005).

Although the mechanism of transmission of iridoviruses in natural settings is unknown, it has been shown that sublethally infected amphibians can cause sporadic, recurrent disease outbreaks in amphibians (Brunner *et al*., 2004). Experimentally and naturally infected tiger salamander larvae and metamorphs were able to maintain sublethal, transmissible infections for over five months. Apparently healthy infected dispersing metamorphs were returning to water bodies to breed and it was speculated that these individuals were likely serving as a reservoir host for infecting newly hatched larvae, creating recurrent outbreaks of disease. The current study showed that turtles may also become asymptomatic carriers, although further studies would help to confirm this finding. Turtle No. 8 which was inoculated intramuscularly showed transient signs of disease but then recovered. At necropsy, tissues collected from eight different organs were negative for iridovirus on PCR. Kidney samples inoculated onto TH-1 cells showed no cytopathic effects. However, cloacal swabs collected one, four and eight days prior to necropsy were positive using PCR. If this was a result of slow elimination of the virus, all orally inoculated turtles should have had PCR-positive cloacal samples after 2 DPI.

However, all swabs taken after this time period were negative. Furthermore, given that IM inoculation was used in turtle No. 8, any virus shed from the GI tract would require transportation through the blood stream and across the wall to be there. While it is possible that this was a laboratory contaminant, all swabs were extracted and tested by PCR according to date collected as opposed to all swabs tested at the same time per turtle. If there was contamination, we would not expect to see it on five occasions from one turtle and not from any orally inoculated turtles on any date past 2 DPI. Therefore it seems more likely that virus shedding was occurring, and the site of virus replication was missed. Some viruses show a predilection for specific cells, such as infectious bursal disease virus in chickens, where virus shows a predilection for the cells of the bursa of Fabricius, located in the cloaca (Burkhardt and Müller, 1987). Further long-term studies would help to confirm whether *Ranavirus* persistence occurs and immunohistochemical or *in situ* hybridization studies of tissues from infected turtles may help identify the tissue tropism for *Ranavirus* persistence. If turtles can serve as asymptomatic carriers, they may also serve as a reservoir host of virus for other turtles and other susceptible species. Inter-class infections of iridovirus have been shown naturally and experimentally in sympatric species of fish and amphibians, where both were capable of being infected with the same virus (Mao *et al*., 1999, Moody and Owens, 1994). In another study (data not shown), the isolate used in this experimental study was capable of infecting leopard frogs (*Rana pipiens*) that were injected intraperitoneally at the same dose. Thus, sublethally infected turtles such as one of the turtles (No. 8) in our study could serve as a reservoir host for amphibian populations in geographic locations where the species overlap.

In summary, the experimental inoculation of a *Ranavirus* in chelonians was investigated. Koch's postulates were fulfilled when intramuscular inoculation of virus into naive turtles resulted in clinical and histologic changes consistent with those seen in natural infections, and when the same virus was subsequently recovered. Since oral inoculation failed to result in disease or mortality, the natural route of transmission in the wild remains unknown. The immune system of reptiles is temperature dependent (Cooper *et al*., 1985) and perhaps by manipulating the environmental temperature, and the temperature of the host, the susceptibility of turtles to infection can be altered. Another possibility that should be investigated is the transport and inoculation of *Ranavirus* into chelonians by arthropods. This study also suggests that sublethally infected turtles may serve as reservoir hosts of infection for other chelonians, as well as amphibians. Ranaviruses are considered a global threat to amphibian populations based on the lack of host specificity, high virulence and global distribution (Daszak *et al*., 1999) and this study confirms that they should likewise be considered a threat to chelonian populations.

Table 4-1. PCR results on tissues collected at necropsy from the pilot study box turtles (BT; *Terrapene ornata ornata*) and red-eared sliders (RES; *Terrapene scripta elegans*) in the full transmission studies.

	Controls				Orally Inoculated Turtles						IM Inoculated Turtles					
	Pilot Study		Full Study		Pilot Study		Full Study				Pilot Study		Full Study			
Tissue	P1 (BT)	P4 (RES)	4	9	P2 (BT)	P5 (RES)	1	2	7	10	P3 (BT)	P6 (RES)	3	5	6	8
Liver	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Kidney	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Spleen	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Tongue	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Esophagus	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Stomach	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Small Intestine	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Large Intestine	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+

Table 4-2. Polymerase chain reaction (PCR) results of oral and cloacal swabs taken on eleven different days post-inoculation (DPI) with an uninfected (control) or iridovirus infected cell lysate throughout the four-week full transmission study. O = oral, C=cloacal. [a] "N/A", indicates the turtle was euthanized prior to the sample date.

	Oral Control		IM Control		Red ear sliders inoculated orally								Red ear sliders inoculated intramuscularly							
I.D.	4		9		1		2		7		10		3		5		6		8	
DPI	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C	O	C
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	+
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+
9	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	+	+	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	N/A	N/A	-	-
16	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	N/A	N/A	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	N/A	N/A	-	+
21	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	N/A	N/A	-	-
23	-	-	-	-	-	-	-	-	-	-	-	-	+	+	N/A	N/A	N/A	N/A	-	+
26	-	-	-	-	-	-	-	-	-	-	-	-	N/A[a]	N/A	N/A	N/A	N/A	N/A	-	+

Table 4-3. PCR results for urine collected opportunistically from turtles in the full transmission study.

	Controls		Orally Inoculated Turtles				IM Inoculated Turtles			
Days p.i.	4	9	1	2	7	10	3	5	6	8
0	N/A	-	-	-	-	-	-	-	-	N/A
5	-	-	N/A	-	-	-	-	-	-	-
7	-	-	-	N/A	-	-	-	-	+	N/A
8	N/A	-	N/A	-	-	-	N/A	-	+	-
11	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+	N/A	N/A
12	N/A	-	N/A	N/A	-	-	N/A	N/A	+	-
15	N/A	-	-	-	-	-	-	N/A	N/A	N/A
19	-	-	-	N/A	-	-	N/A	N/A	N/A	-
23	N/A	N/A	N/A	N/A	N/A	N/A	+	N/A	N/A	N/A
27	-	-	-	-	-	-	N/A	N/A	N/A	-

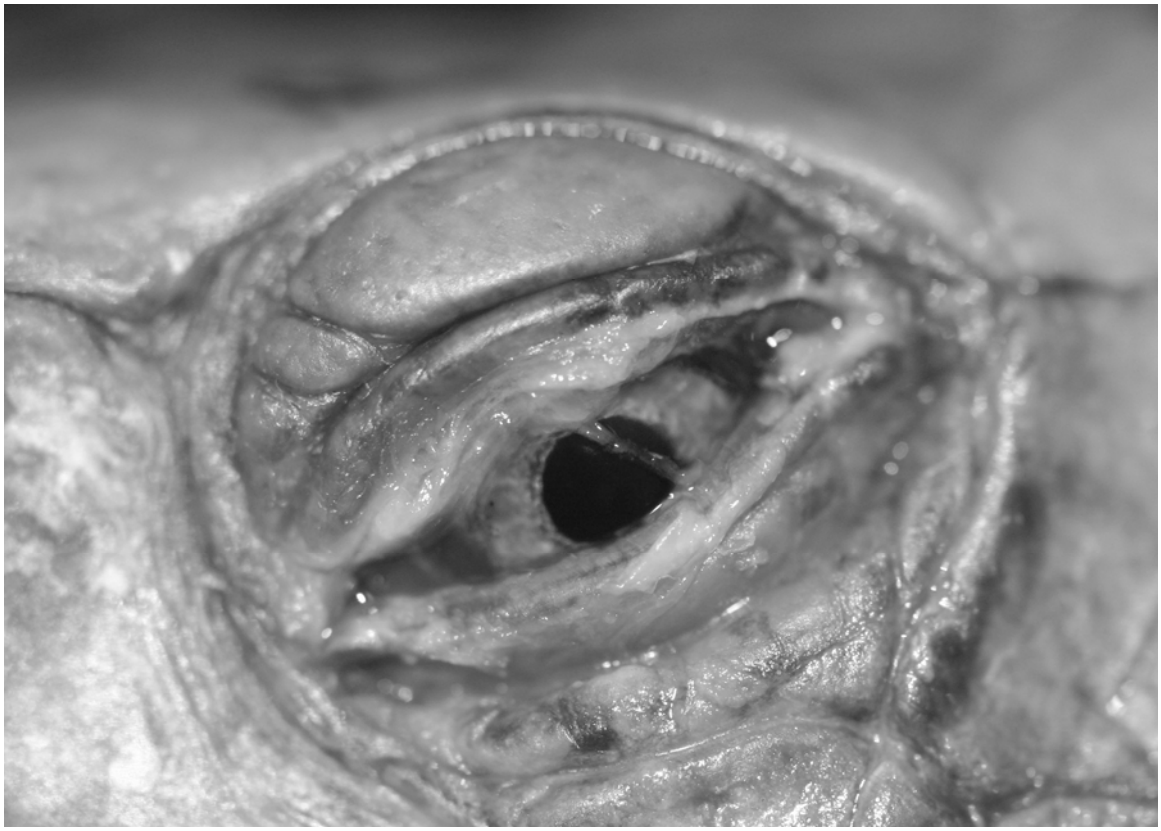


Fig. 4-1. Photograph taken 12 days post-inoculation showing development of white opaque ocular discharge in the IM inoculated box turtle (*Terrapene ornata ornata*).

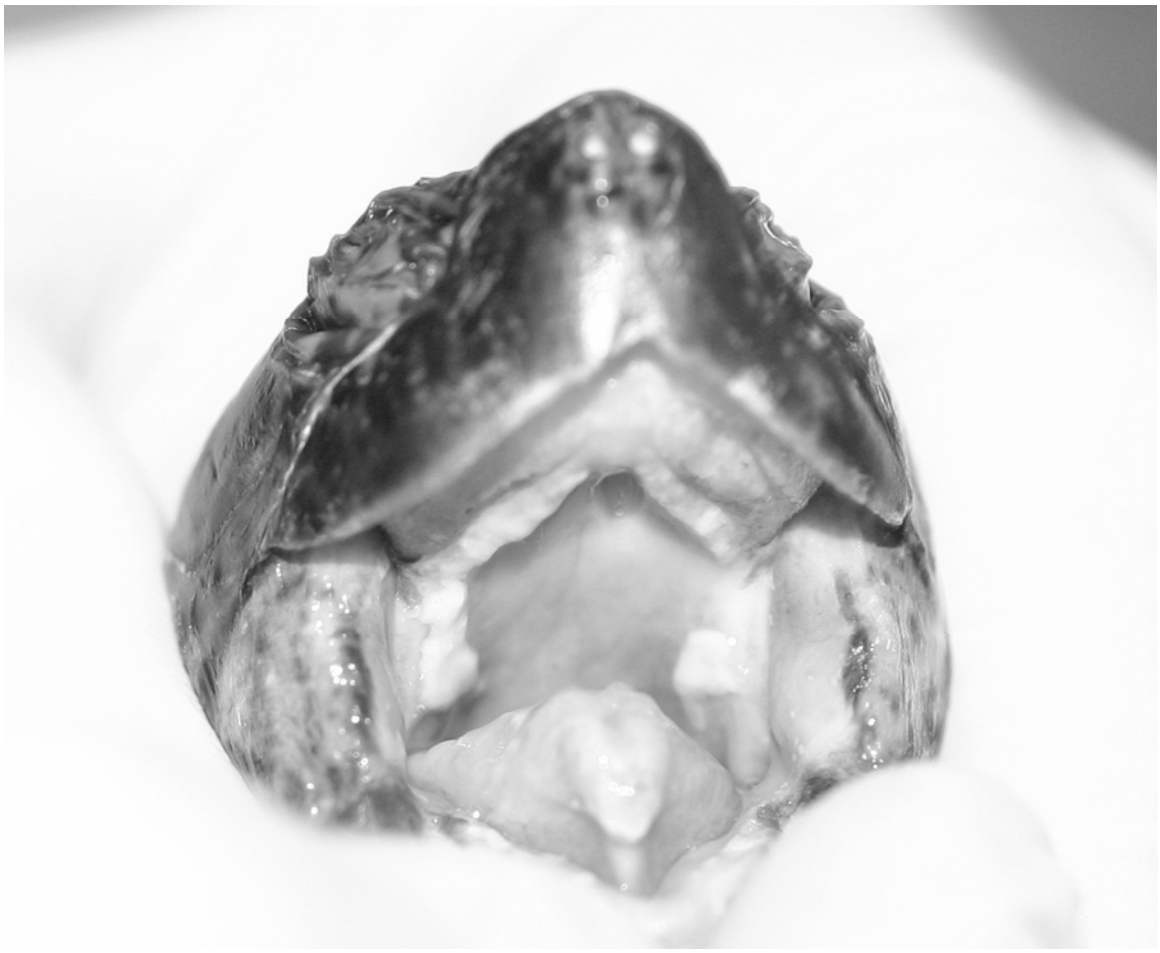
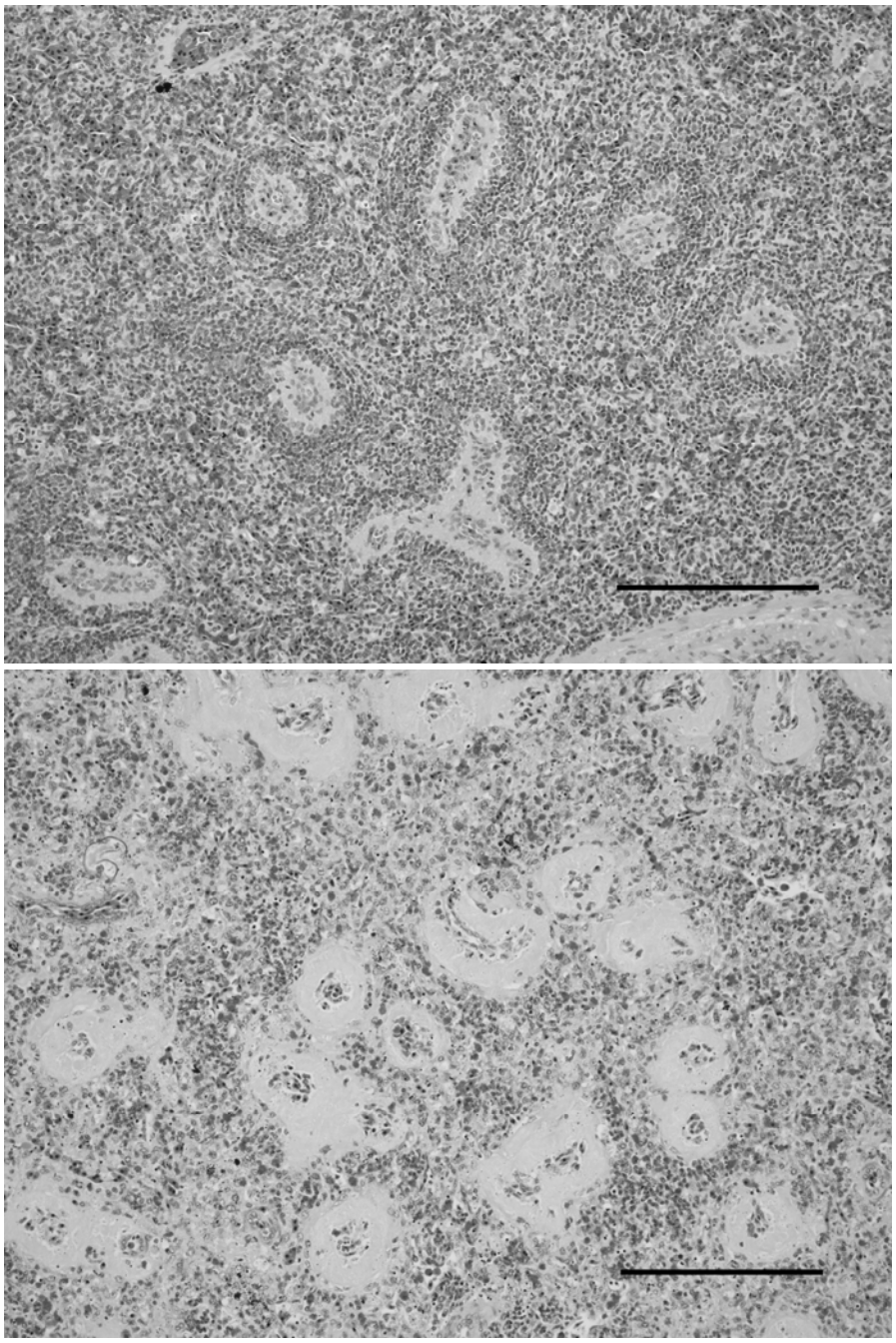


Fig. 4-2. Photograph taken 12 days post-inoculation showing white caseous diphtheric plaques in the mouth of an IM inoculated red-eared slider (*Trachemys scripta elegans*).

Fig. 4-3. Photograph showing exophthalmus, conjunctivitis and hyphema in an intramuscularly inoculated red-eared slider (*Trachemys scripta elegans*).

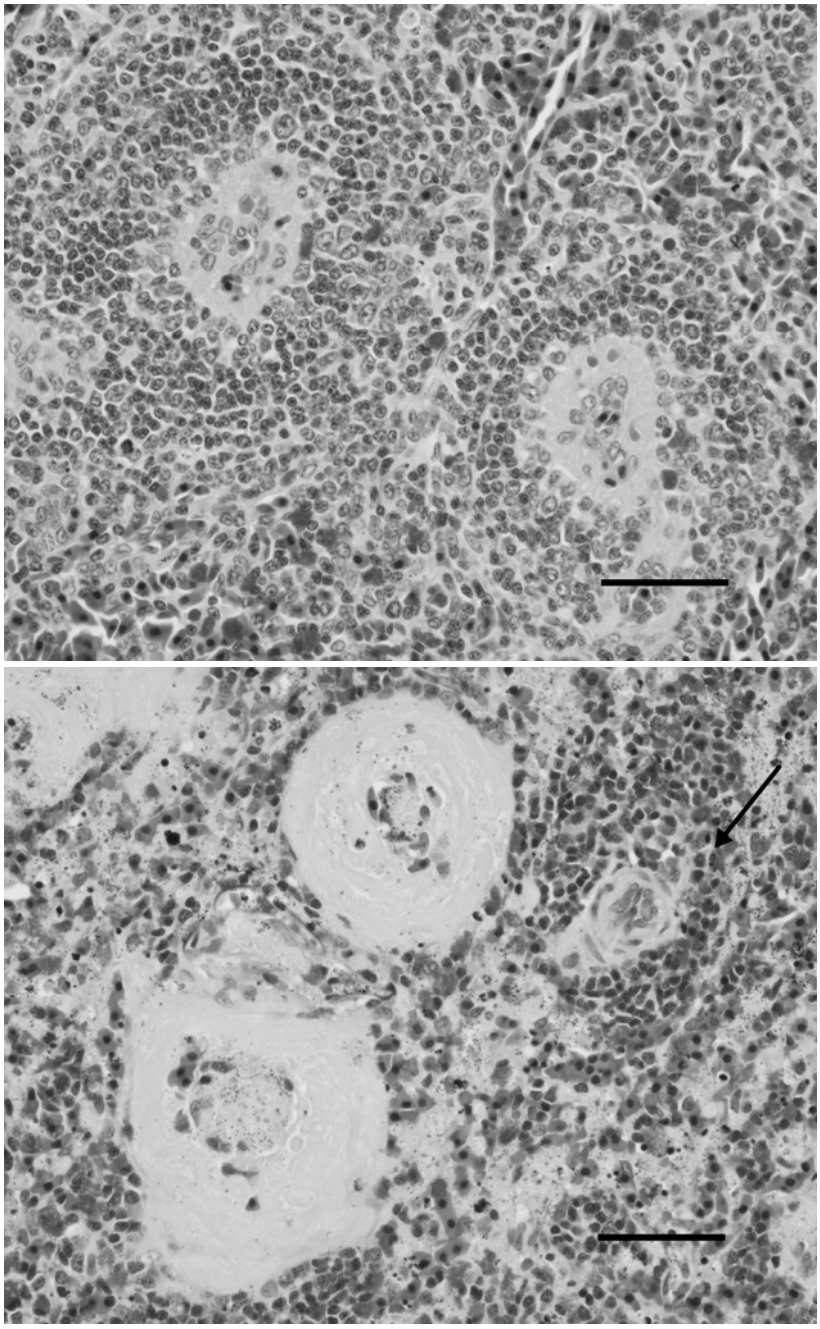
Fig. 4-4. Photograph showing colonic hemorrhage in a turtle intramuscularly inoculated with *Ranavirus* euthanized 23 days post inoculation.



A

B

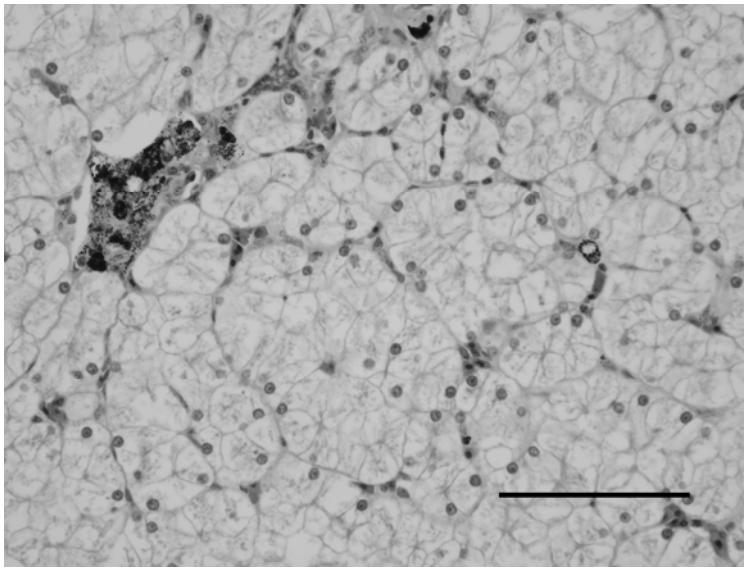
Fig. 4-5. Spleen; red-eared slider (*Trachemys scripta elegans*). A) Sham inoculated turtle. The white pulp is concentrated in discrete cuffs around splenic ellipsoids. H&E stain. Bar = 200 *u*m. B) Turtle intramuscularly inoculated with *Ranavirus*. There is lymphoid depletion and expansion of the walls of splenic ellipsoids by eosinophilic material. H&E stain. Bar = 200 *u*m.



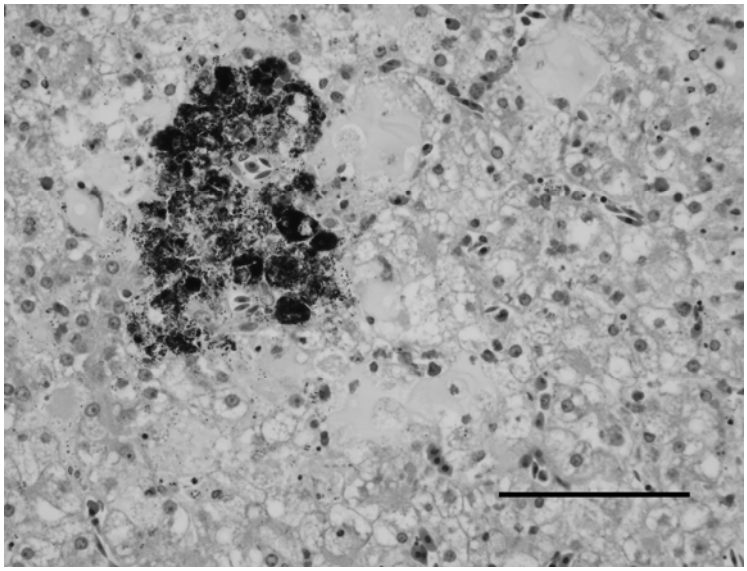
A

B

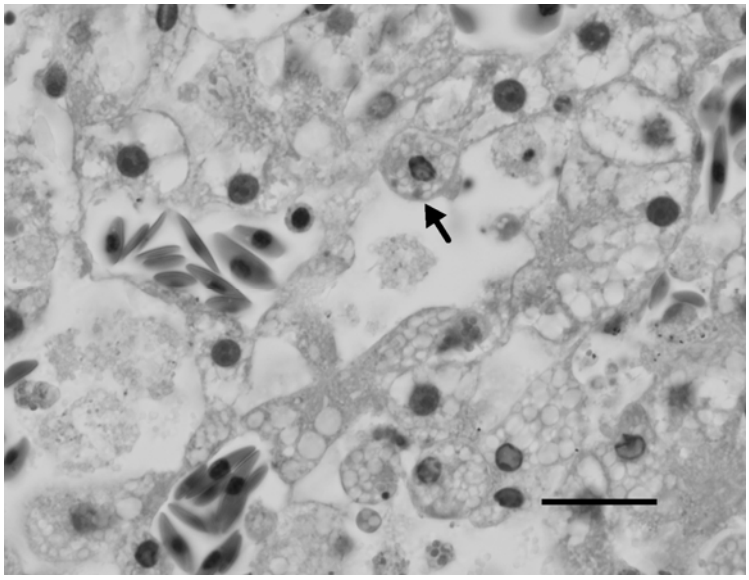
Fig. 4-6. Spleen; red-eared slider (*Trachemys scripta elegans*). A) Sham inoculated turtle. A higher magnification of two splenic ellipsoids and associated periellipsoidal lymphocyte sheathes (PELS). H&E stain. Bar = 50 *u*m. B) Turtle intramuscularly inoculated with *Ranavirus*. Higher magnification of two splenic ellipsoids and one splenic arteriole (arrow). The walls of the ellipsoids are expanded by fibrin and there are lumenal fibrin thrombi. Note the sparing of the splenic arteriole and associated lymphoid sheath (periarteriolar lymphoid sheath (PALS). H&E stain. Bar = 50 *u*m.



A

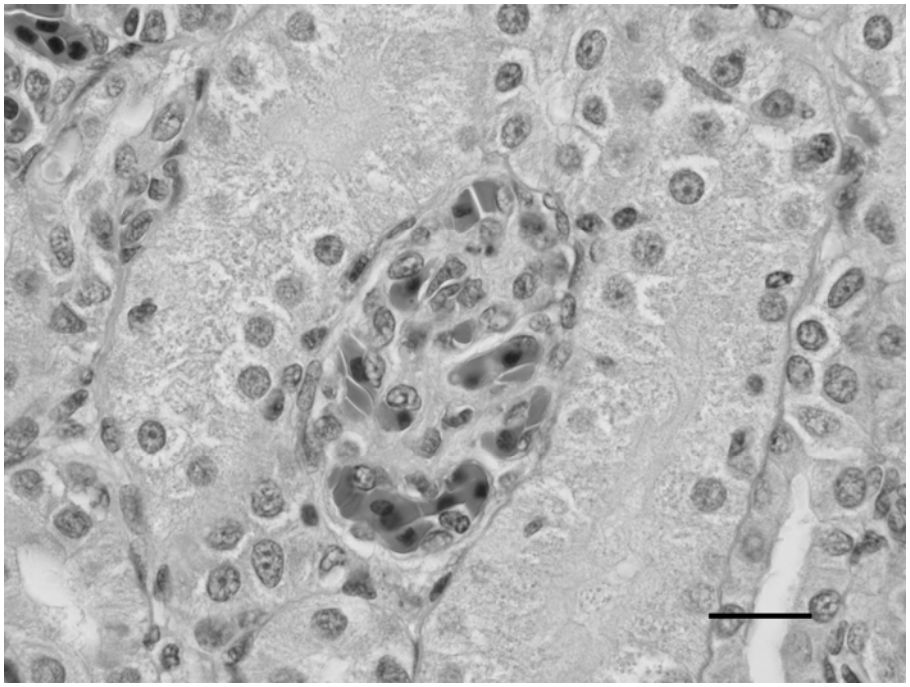


B

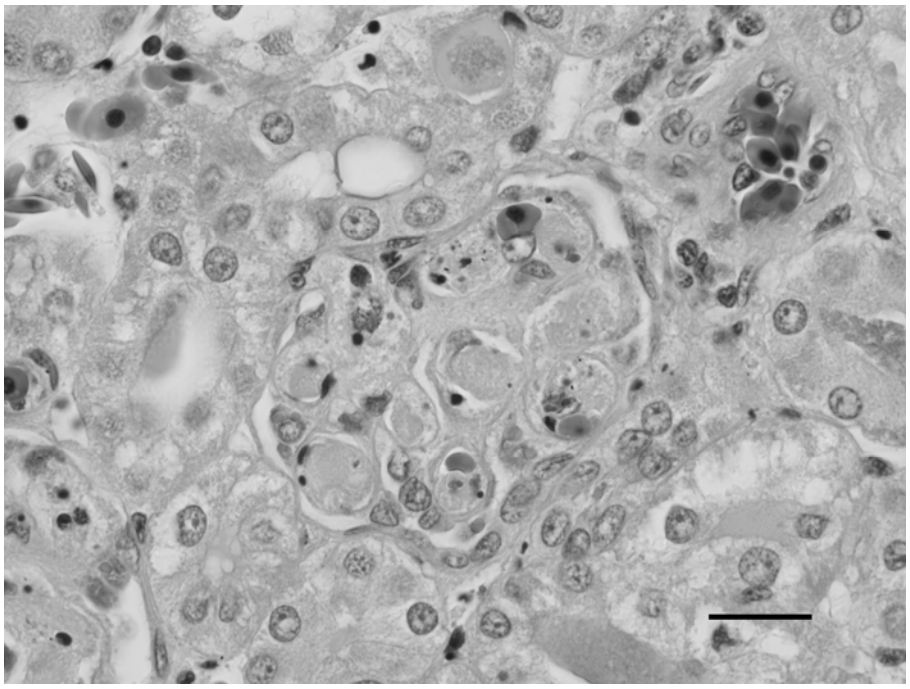


C

Fig. 4-7. Liver; red-eared slider (*Trachemys scripta elegans*). A) Sham inoculated turtle. There is marked diffuse vacuolation of hepatocytes. A melanomacrophage center is present in the upper left. H&E stain. Bar = 100 *u*m. B) Turtle intramuscularly inoculated with *Ranavirus*. There is depletion of normal hepatocellular lipid and glycogen. Sinusoids are multifocally expanded by fibrin thrombi with infiltrates of low numbers of heterophils. H&E stain. Bar = 100 *u*m. C) Turtle intramuscularly inoculated with *Ranavirus*. There is single cell necrosis of hepatocytes with occasional intracytoplasmic basophilic inclusion bodies (arrow). H&E stain. Bar = 20 *u*m.

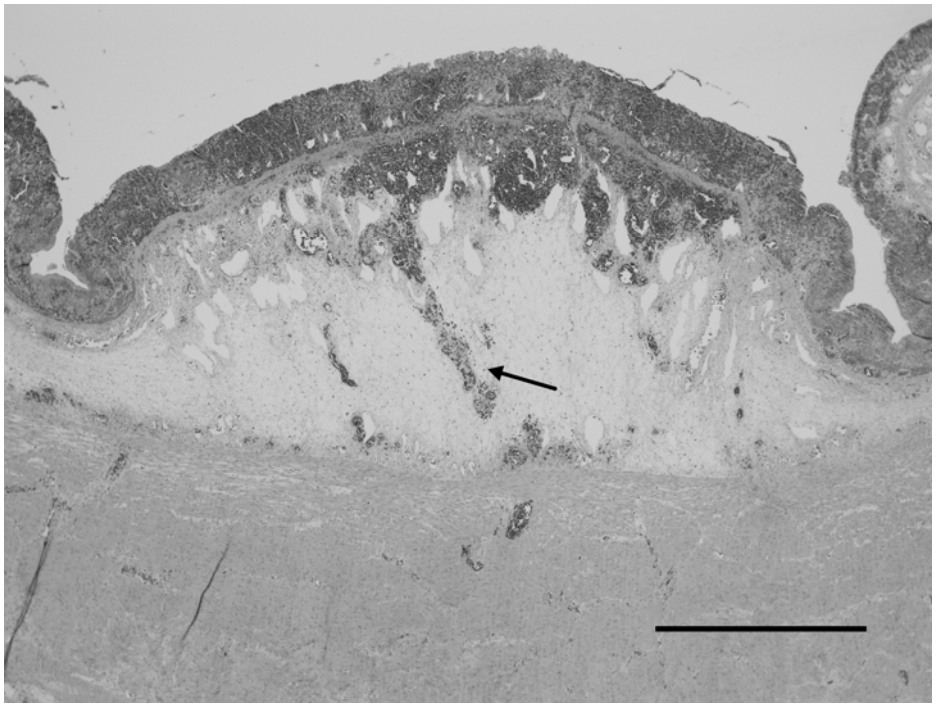


A

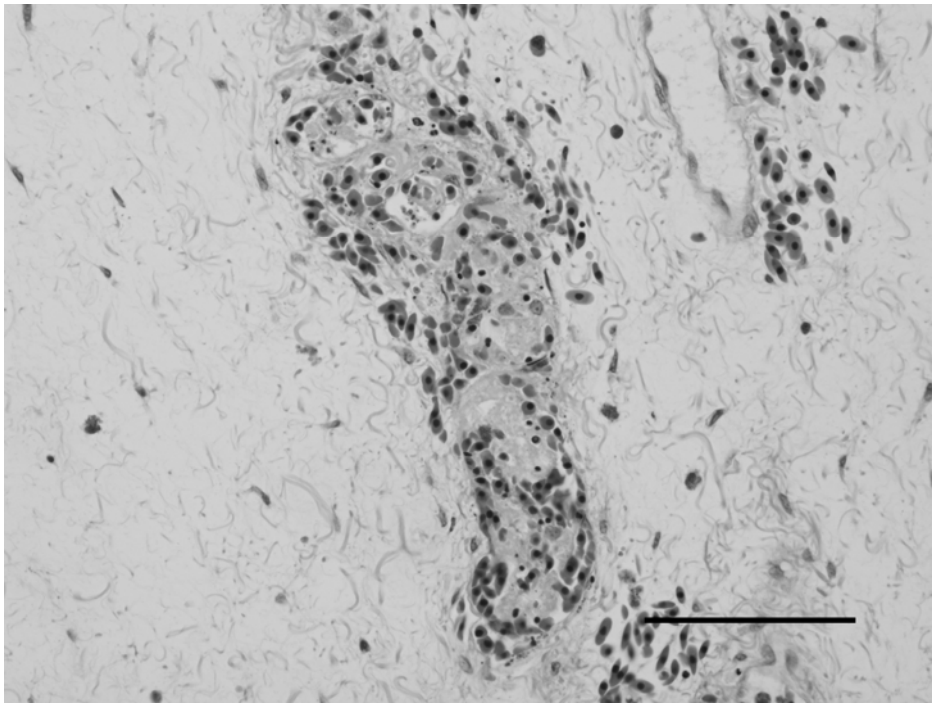


B

Fig. 4-8. Kidney; red-eared slider (*Trachemys scripta elegans*). A) Sham inoculated turtle. Normal glomerulus. H&E stain. Bar = 20 *u*m. B) Turtle intramuscularly inoculated with *Ranavirus*. There are fibrin thrombi in glomerular capillary loops admixed with few heterophils and small amounts of karyorrhectic debris. H&E stain. Bar = 20 *u*m.



A



B

Fig. 4-9. Colon; red-eared slider (*Trachemys scripta elegans)* intramuscularly inoculated with *Ranavirus*. A) There is mucosal and superficial submucosal hemorrhage with marked submucosal edema. The arrow indicates a submucosal blood vessel. H&E stain. Bar = 1.0 mm. B) Higher magnification of submucosal blood vessel shown in 4-5A. There is a lumenal fibrin thrombus with admixed karyorrhectic debris and mild perivascular hemorrhage. H&E stain. Bar = 100 *u*m.

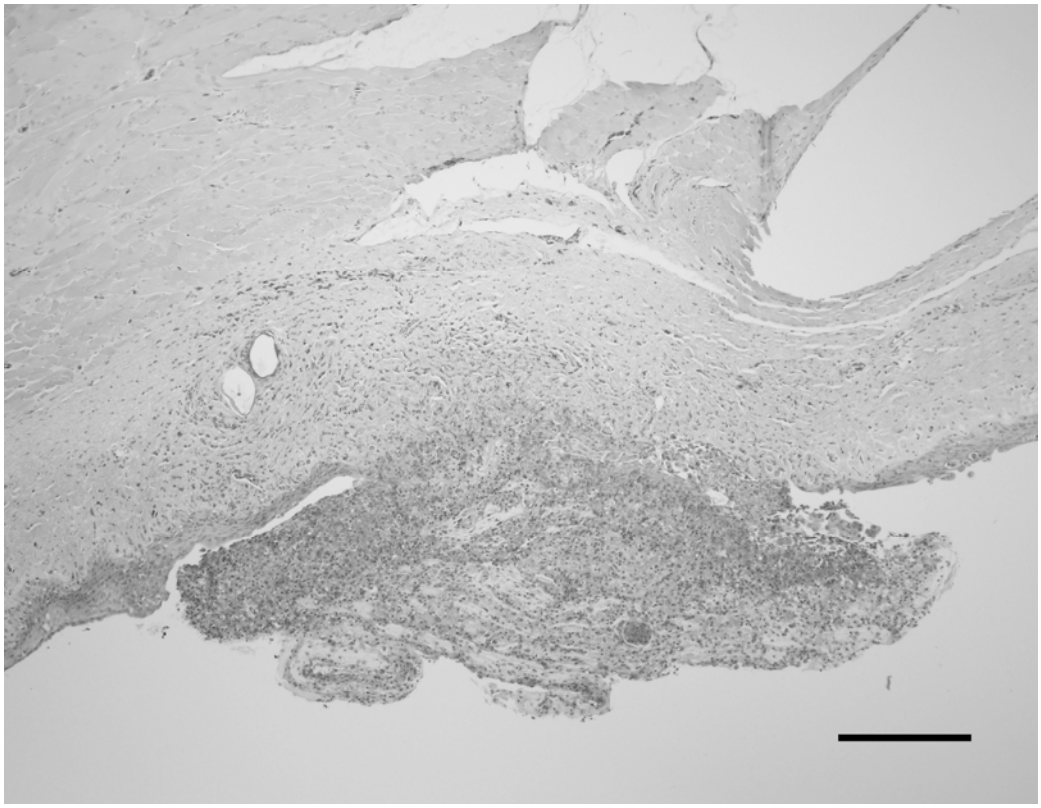


Fig. 4-10. Oral mucosa; red-eared slider (*Trachemys scripta elegans*) intramuscularly inoculated with *Ranavirus*. There is focal mucosal ulceration and replacement by a mat of heterophils and fibrin. H&E stain. Bar = 200 *u*m.

CHAPTER 5
THE ROLE OF INFECTED LEOPARD FROGS (*Rana pipiens*) IN TRANSMISSION OF A RANAVIRUS IN RED-EARED SLIDERS (*Trachemys scripta elegans)*

Introduction

Emerging infectious diseases have been increasingly recognized as factors influencing wildlife health and populations (Harvell *et al*., 1999; Daszak *et al*., 2000). Iridoviruses in the genus *Ranavirus* are being observed at an increasing frequency in chelonians throughout the eastern United States (DeVoe *et al.,* 2004; Johnson *et al.,* 2004). While the route of transmission of this virus to chelonians has yet to be identified, it is suspected that amphibians may be a reservoir host.

A Burmese star tortoise (*Geochelone platynota*) with clinical signs of illness (ocular, nasal and oral discharge, palpebral and cervical edema, and stomatitis) died at a captive breeding facility in Georgia. Molecular techniques including polymerase chain reaction and nucleotide sequencing demonstrated the presence of *Ranavirus* major capsid protein genes in various tissues. Transmission electron microscopy of tissues and cell cultures inoculated with tissue homogenate of kidney from this tortoise demonstrated the presence of virions consistent in shape and size with iridoviruses (Johnson *et al.,* 2004).

Tortoises in an adjacent pen were observed ingesting dead amphibians, and so, frogs and toads from the site of the tortoise death were collected and euthanized to determine if they were infected with *Ranavirus*. One frog was found to be positive by PCR, with the amplicon sharing 100% identity across approximately 500 basepairs with the Burmese star tortoise isolate (BSTRV) as well as Frog Virus 3, the type species of the

genus *Ranavirus*. Histologic lesions consistent with amphibian *Ranavirus* infection were observed in this frog (Johnson *et al.,* 2004). Virus was isolated and whole viral genomic restriction utilizing two endonucleases comparing the frog and tortoise isolates showed identical restriction patterns, suggesting that they were either infected with the same or very closely related strains of the same virus.

Experimental transmission studies fulfilling Koch's postulates were performed confirming the BSTRV isolate is a causative agent of disease and mortality in experimentally inoculated western ornate box turtles (*Terrapene ornata ornata*) and red-eared sliders (*Trachemys scripta elegans*). Mortality was only observed when turtles were inoculated intramuscularly and not when turtles were inoculated orally. This is different than what has been shown with experimentally inoculated amphibians, where ingestion of infected water and infected amphibian conspecifics resulted in infection and increased mortality rates (Jancovich *et al*., 2001, Pearman *et al*., 2004). One hypothesis for why this occurred was that the natural route of exposure was not being replicated in the laboratory. Turtles were inoculated with an infected cell lysate by gavage feeding tube into the distal esophagus, when in a natural setting they would be ingesting bones and other organic matter that might cause natural abrasions in the gastrointestinal tract, allowing a route of entry of the virus into the blood stream. Turtles in the wild might also be subjected to multiple exposures of virus, as opposed to a single dose as was administered in the previous transmission study.

To attempt to establish a route of transmission that might be occurring in natural settings, leopard frogs were experimentally inoculated with BSTRV, euthanized, homogenized and fed to turtles in an attempt to determine if ingestion of infected

amphibians might be responsible for infection of chelonians in the wild. We evaluated infection by observing for clinical signs seen in chelonians infected with *Ranavirus*, as well as seroconversion using an indirect ELISA. Here we report the results of this study.

Materials and Methods

Virus Preparation

A previously isolated and partially characterized *Ranavirus* from a Burmese star tortoise (BSTRV; *Geochelone platynota*; Johnson *et al.*, 2004) was used to inoculate frogs in this study. Briefly, polymerase chain reaction targeting a portion of ranaviral major capsid protein genes followed by DNA sequencing demonstrated that the BSTRV isolate shared 100% sequence identity with Frog Virus 3, the type species for the genus *Ranavirus* in the family Iridoviridae. Restriction enzyme digests of BSTRV compared with FV3 showed identical restriction patterns using two enzymes, *Hind*III and *Xba*I, indicating that BSTRV is either identical or closely related to FV3.

Terrapene heart cells (TH-1) were acquired from the American Type Culture Collection (ATCC-CCL 50; Rockville, MD) and grown to confluency in 225cm^2 tissue flasks (Costar, Corning, NY). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60mg/liter; Sigma, St. Louis, MO), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300µg/liter; Sigma). Cells were inoculated with a fourth passage of BSTRV and incubated at 28ºC in the presence of 5% CO_2. When cytopathic effect (CPE) was observed, consisting of cell rounding and detachment from the flask in over 70% of cells, the flasks were scraped and contents transferred to 15 ml centrifuge tubes and clarified by slow speed centrifugation at 4,500x*g* for 30 minutes. The supernatant was then discarded and the cell pellet

resuspended in 10mls of cell culture media. The preparation was then vortexed, frozen and thawed three times to release virus from the cells into the supernatant. The preparation was again clarified by centrifuging for thirty minutes. The supernatant was then transferred to a new tube and the cell pellet was discarded. The live virus in the media was then quantified by a $TCID_{50}$ assay, and frozen at -80°C until use.

Frog Pilot Study

A pilot study was undertaken to determine the time post-inoculation that would be ideal for maintaining the highest titer of virus in infected frogs to be used in a feeding study. This and the following study were performed under the approval of the Institutional Animal Care and Use Committee at the University of Florida. Thirteen wild caught adult Rio Grande leopard frogs (*Rana berlandieri*) were obtained from a biological supply company. Frogs averaged 72 grams and were placed in groups of three or four in plastic containers on damp moss. A shallow water dish was available for soaking. Oral and cloacal swabs were collected from each individual upon arrival and assayed for the presence of iridovirus using a polymerase chain reaction (PCR) as previously described (Mao *et al*., 1997) and further described below. Once all frogs were determined to be negative, nine frogs in three containers were inoculated with the BSTRV isolate. Each frog received 0.5mls of a $10^{6.4}TCID_{50}$/ml infected cell lysate in its ventral lymph sac (subcutaneously; Fig. 5-1). The container housing four frogs remained uninfected as controls for normal tissue for histopathology and to see if any of the four were positive on PCR once tissues were assayed.

One container of three inoculated frogs each was euthanized on days 3, 7 and 14 post-inoculation, and the four controls were euthanized 14 days post-inoculation. Frogs were euthanized by an overdose of CO_2 followed by decapitation after a loss of righting

abilities was observed and there was no corneal reflex or response to painful stimuli. A small piece of liver was collected and frozen at -80°C for PCR, while another portion was placed in 10% phosphate buffered formalin for histopathology. A small piece of spleen was collected and frozen for virus isolation and determining virus titer differences between individuals and between post-inoculation days.

Full Frog Study and Pre-inoculation Sampling

Seventy-one captive raised leopard frogs (*Rana pipiens*) were obtained from a biological supply company and placed in ten groups of seven or eight in plastic containers in an animal care facility room at the University of Florida. Oral and cloacal swabs were collected from each frog, and combined into one tube. Frogs were identified only by container and not individually at this stage. DNA was extracted from the swabs using the DNeasy kit (Qiagen, Valencia, CA) and assayed by PCR for the presence of iridovirus. Sense primer (5'-GACTTGGCCACTTATCAC-3') and anti-sense primer (5'-GTCTCTGGAGAAGAAGAA-3') as previously described (Mao *et al*., 1997) were used to amplify approximately 500 base pairs of the *Ranavirus* major capsid protein gene. A 20 μl reaction mixture was run which contained 2μl extracted DNA, 1 μM of each primer, 200 μM each of dATP, dCTP, dGTP, and dTTP, 1.5 U of *Taq* DNA polymerase and PCR buffer containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$ (Eppendorf, Westbury, New York, USA). The mixtures were amplified in a thermal cycler (PCR Sprint, Thermo Hybaid) with an initial denaturation at 94˚C for 2.5 min, followed by 25 cycles of denaturation at 94˚C for 30 sec.; annealing at 50˚C for 30 sec, extension at 72˚C for 30 sec., and a final extension step at 72˚C for 10 min as previously described (Marschang *et al*., 1999). Two frogs were positive upon arrival by PCR in

containers 3 and 6. All seven frogs in container six were euthanized as described for the pilot study and identified by individual and container (6-1 through 6-7). A portion of the spleen was removed on each and set up for PCR. The carcasses were refrigerated until the PCR results were determined. Frog 6-4 was found to be positive for iridovirus, and it was placed in formalin and submitted for histopathology. The remaining frogs were frozen at -80°C.

Thirty frogs in containers 5, 7, 8, 9, and 10 were experimentally inoculated with 500µl of a$10^{5.75}$ $TCID_{50}$/ml virus culture injected into their ventral lymph sacs. Frogs in containers 1-4 remained uninoculated. Seven days post-inoculation all the frogs were euthanized by CO_2 as described for the pilot study. A small piece of liver was collected for PCR to ensure the frogs were either infected or that control frogs were uninfected. Organs were removed from each frog and stored frozen at -80°C in sealed plastic storage bags until PCR could be performed. Inoculated frogs that had strong positive signals by PCR and control frogs that were not inoculated and negative by PCR were used for feeding to turtles. Organs were separated and homogenized in a food processor. Four skeletons were added to each group to provide bits of bone and objects normally encountered when eating a carcass. The homogenized frogs were stored frozen at -80°C in 35ml aliquots for feeding (slightly more than the amount deemed necessary to feed all eight turtles in the feeding group for one day).

Turtles

Sixteen red-eared sliders were obtained from an animal dealer. Seven were adults (Nos. 1-7), while nine were juveniles (Nos. 8-16). Size differences were a result of availability at the time of the study. Upon arrival turtles were randomly assigned

numbers within their size groups and anesthetized with intravenous propofol. A pharyngostomy tube was inserted through the cervical skin, and with the inserted end passed to the esophageal-gastric junction. The tube was held in place using a Chinese mattress suture pattern (Fig. 5-2). Feeding tube sizes were selected such that the largest tube, as deemed appropriate for the size of the turtle, was used in order to reduce the chance of the tube clogging and to allow larger particles of food such as bone bits to pass through the tubes. The seven adults were randomly assigned to one of two feeding groups, and the juveniles were similarly randomly separated into the two groups. Eight turtles (Nos. 1, 3, 4, 5, 8, 11, 12 and 13) in group 1 received virus inoculated frog homogenate, while eight turtles in group 2 (Nos. 2, 6, 7, 9, 10, 14, 15, and 16) received uninfected homogenized frogs.

Aliquots of food were thawed within three hours of the time of feeding and mixed with equal volumes of water and mixed well. This was sufficient for passing through the tubes of the adult turtles using 10ml syringes, but was too thick for passing through feeding tubes of juveniles. The food for juveniles were mixed 2:1 of water to food, well mixed and then filtered through sterile gauze to filter out larger particles. Turtles were then administered food with 3ml syringes. Turtles were fed 1% of their total body weight in food at each feeding, twice weekly for six weeks. Water was administered into the feeding tubes after food was given to ensure the full frog meal was out of the tube and in the gastrointestinal tract and to keep the tubes patent.

Physical examinations were performed daily to assess the presence of the following clinical signs: lethargy, anorexia, cervical edema, palpebral or periocular edema, ocular discharge, nasal discharge, oral discharge, the presence of oral plaques or any other

abnormalities. Any turtle found to have severe clinical signs of disease was euthanized. Blood was collected from the subcarapacial vein from each turtle prior to inoculation and once a week for the three-month study. Blood was centrifuged and plasma collected for evaluation of production of anti-*Ranavirus* antibodies by ELISA.

Full necropsies were performed on all turtles at the time of death, euthanasia or at the end of the three-month study. For euthanasia, ketamine was administered intramuscularly at 100 mg/kg followed by intravenous sodium pentobarbital. Once turtles were unresponsive to painful stimuli and showed no corneal reflex, they were decapitated and a complete necropsy performed. Portions of tongue, esophagus, stomach, small and large intestine, liver, kidney, and spleen were collected aseptically by flame sterilizing tools between each organ, and frozen at -80°C for virus isolation and/or DNA extraction and PCR. The following tissues were collected and fixed in 10% neutral phosphate buffered formalin: tongue, esophagus, stomach, small and large intestine, liver, kidney, spleen, pancreas, heart, trachea, lung, brain, urinary bladder, thyroid gland, adrenal gland, bone, bone marrow, skin, skeletal muscle, nasal cavity, eye and gonad. These tissues were then processed for histologic examination. They were embedded into paraffin, and 6 μm sections were stained with hematoxylin and eosin.

Virus Titers

A $TCID_{50}$ assay as previously described was performed on the homogenized frogs at both the 1:1 and the 2:1 dilutions, before the first feeding and then again at 2 weeks and 4 weeks to determine if any reduction in titer was occurring as a result of being stored in the freezer over time. For both the 1:1 and 2:1 dilutions, 100µl was taken, diluted in cell culture media and syringe filtered using a 0.45µm filter (Costar, Corning,

NY) into 9.9mls of media. This 1:10 dilution was then ten-fold serially diluted. One ml of each dilution was placed into four wells of a 24 well plate from 10^{-2} to 10^{-7}. The plates were incubated for 5 days at 28°C and then observed for the presence of CPE. The $TCID_{50}$ was then calculated for each type of food.

ELISA

An indirect enzyme linked immunosorbent assay (ELISA) was used to determine the presence of anti-*Ranavirus* antibodies. The ELISA methodology was similar to that developed for use in identifying the presence of anti-tortoise herpesvirus antibodies in tortoises (Origgi *et al*., 2004) and anti-west Nile virus antibodies in alligators (Jacobson *et al*., 2005). The BSTRV isolate was used as the antigen in the assay. Each well of a 96 well high-protein binding microplate was coated overnight at 4 ^{o}C with 50 µl of a 1:100 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 BSTRV. Lysates were diluted in 0.01 M sodium phosphate buffer (pH7.2) containing 0.15 NaCl and 0.02% NaN_3 (PBS/A). Wells were then washed four times in ELISA wash buffer (PBS/A 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 were incubated for one hour at room temperature. Plasma samples were added in 50µl volumes at a 1:100 dilution in blocking buffer. The secondary antibody used was a biotin-conjugated mouse anti-tortoise immunoglobulin (Ig) monoclonal antibody diluted to a final concentration of 0.5µg/ml in blocking buffer. Alkaline phosphatase-conjugated-streptavidin (Zymed Laboratories, Inc., San Francisco, CA) was then applied

to each well at 50 µL of a 1:5000 dilution in PBS/A. Next, 100 µl of a 1.0 mg/ml P-nitrophenyl phosphate prepared in 0.01 M sodium bicarbonate buffer containing 2 mM $MgCl_2$ was added to each well and the plates were then stored in the dark until being read. The optical density (OD) of each well was read at A_{405} using a StatFax 3200 microplate reader (Awareness Technology, Palm City, Florida, USA) after 30 minutes.

Each sample was done in triplicate: one time on wells coated with uninfected cell lysate and in duplicate on wells coated with infected cell lysate. The replicate values of the wells coated with BSTRV lysate were averaged and divided by the OD reading of the value of the plasma sample run on the uninfected cell lysate to subtract out any background binding that might be caused by cross-reactivity to the cells. This makes up the positive to negative ratio (P/N ratio). Ratios greater than 2.2 were considered positive as previously determined by taking the mean P/N ratio of 1000 free-ranging gopher tortoise samples plus three times the standard deviation of the mean.

Results

Frog Pilot Study

Of the three frogs euthanized three days post-inoculation, only one frog liver was positive for the presence of iridovirus on PCR (Table 1). Light microscopic observation of a portion of liver of this frog demonstrated mild, multifocal sinusoidal karyorrhectic debris. The other two frogs were PCR negative and showed no histologic changes (Fig. 5-3). At seven days post-inoculation, one frog died naturally and two others were euthanized. The frog that died naturally had multifocal necrosis (Fig. 5-4) with basophilic cytoplasmic inclusion bodies in the hepatocytes (Fig. 5-5; arrows denote inclusion bodies), which is consistent with iridovirus infections. The other two frogs had mild to moderate multifocal sinusoidal karyorrhectic debris with fibrin. DNA samples

extracted from the livers of all three frogs were positive on PCR for the presence of iridovirus (Table 1). One frog was positive 14 days post-inoculation. This frog histologically showed moderate multifocal sinusoidal karyorrhectic debris with moderate marked extramedullary hematopoeisis. The other two inoculated frogs were negative on PCR and histologically were normal. PCR of liver samples from control frogs were negative and frog livers were histologically normal. Virus was isolated on TH-1 cells only from frogs with positive PCR results, while frogs with negative PCR results demonstrated no cytopathic effects (Table 1). Virus titers in positive frogs were determined between groups but did not differ significantly between days. As a result of this data, frogs in the larger study for feeding were euthanized 7 days post-inoculation.

Full Frog Study

One additional control frog was found to be positive for the presence of iridovirus when the liver samples were tested by PCR for a total of three of 71 (4.2%) frogs from the biological supply company being positive on arrival for iridovirus. Twenty-one control frogs were selected for blending for turtle food, along with four skeletons for bone matter. Twenty-two of 30 (73.3%) liver samples from experimentally inoculated frogs were positive by PCR for iridovirus at necropsy. Organs from 18 of the samples showing the strongest signal were utilized for food. Weak positives and negatives were excluded to reduce any dilutional effect. Four skeletons were added for bone matter.

Turtles

Four juvenile turtles were euthanized or died prior to the termination of the study. Turtle 9 and 23 died 1 week post-inoculation (WPI), turtle 11 was euthanized two WPI, and turtle 8 was euthanized 4 WPI. At necropsy, it was observed that the pharyngostomy tubes had perforated the esophagus and that all four turtles had likely died as a result of

sepsis from injecting the frog homogenates directly into the coelomic cavity. Three of the turtles were in the infected group and one was in the control group. PCR on tissues collected at necropsy failed to identify iridovirus in two of the turtles being fed *Ranavirus*-infected frogs. One turtle (No. 11) was positive, but because food was being administered unintentionally into the coelomic cavity, it is difficult to say whether the virus was causing an active infection or rather it was simply residual virus from the last feeding. Histopathologic observations suggest the latter, as lesions typically observed in other naturally and experimentally inoculated turtles were not observed.

All other turtles remained clinically healthy throughout the study. PCR of tissues collected at necropsy were negative in each turtle. No histologic changes were seen that were consistent with lesions seen in naturally or experimentally inoculated turtles. At necropsy, the feeding tubes were in the correct locations and no lesions were seen observed with their presence.

Virus Titers

Gauze filtration of the food being fed to juveniles actually resulted in higher titers. Food assayed for juveniles from the first day of feeding had a titer of $10^{3.75}TCID_{50}$/ml while adult food had a titer of $10^{2.5}TCID_{50}$/ml. Titers taken at 2 weeks and 4 weeks had titers of $10^{3.5}$ and $10^{2.5}TCID_{50}$/ml, respectively.

ELISA

Plasma samples collected weekly over the three-month study failed to detect the production of anti-*Ranavirus* antibodies. P/N ratios did not exceed 1.5 in any turtle at all time points in comparison to a positive control observed at 3.5 (Fig. 5-6).

Discussion

Iridoviruses of the genus *Ranavirus* are emerging as important pathogens of captive and free-ranging chelonians in the eastern United States. When managing disease in populations, one of the more important questions becomes "how is this virus being transmitted?" If a method of transmission can be identified, breaks in the chain or route of transmission become important methods of controlling and preventing the spread of disease within and between populations. Little is known about the transmission of *Ranavirus* to chelonians, although cases of infection are being increasingly reported (Allender *et al.*, In Press; DeVoe *et al.*, 2004; Johnson *et al.*, 2004). Recent findings suggest that amphibians may serve as a reservoir host of infection for chelonians, as a frog isolate collected at the same location as a tortoise isolate shared identical restriction patterns when whole viral genomes were restricted with two different endonucleases (Johnson *et al.*, unpublished data). This study was designed to explore the hypothesis that turtles might become infected with iridovirus by ingesting dead infected amphibians.

Experimental studies with amphibians have shown that amphibians can become infected by cannibalism of infected individuals or ingested water (Jancovich *et al*., 2001, Pearman et al, 2004). This indicates that by either source, infection by an oral route is possible under experimental conditions in amphibians. We were unable to replicate these findings in our study with red-eared sliders, either because we failed to replicate natural conditions of oral inoculation, or that chelonians are infected by a different route than amphibians. By placing feeding tubes, we were able to control the amount of food that the turtles ingested and the frequency with which they ate; however, this also limited the amount of food that was ingested to values established for feeding debilitated anorexic turtles in rehabilitation settings. Also, the size of the particulate matter that was ingested

was limited in size. Wild turtles may ingest higher amounts of food than 1% of their body weight used in this study and thus ingest greater titers of virus as infected amphibians are eaten. Or, viral loads in naturally exposed amphibians may achieve higher levels than those that we induced experimentally in the inoculated leopard frogs. By placing the feeding tubes within the esophagus, we created a fixed diameter for food to pass through that is significantly smaller than the flexible diameter of the esophagus in chelonians. So when turtles are ingesting a dead amphibian, much larger pieces can be ingested, along with bones and other organic matter that might be adhered to the carcass. These bones or other hard matter might cause natural abrasions that we did not replicate in our study, although placement of a feeding tube should have created a breakdown (even if temporary) in the gastrointestinal-vascular barrier. Additionally, we bypassed the oral cavity by placing the feeding tube in the caudal esophagus, which may or may not play an important role in virus-receptor interactions.

Additional factors may have caused us to fail to recognize a true route of oral transmission. Studies with experimental inoculation of iridoviruses in amphibians have shown that both host and dose characteristics are important in determining the mortality rates, as well as the duration of disease (Brunner *et al*., 2005). The infection dose was positively correlated with the mortality rate and inversely related to average survival times. Environmental temperatures have also been shown to significantly impact the percent mortality and time to death in salamanders experimentally inoculated with a *Ranavirus* (Rojas *et al*., 2005), where salamanders inoculated at 18 and 10°C were more likely to die than those exposed at 26°C. While water and room temperatures in this study averaged between approximately 21 and 25°C, basking areas were kept warmer at

28°C. Eliminating heat lamps over basking areas and lowering the room temperature might have kept turtles cooler, and altered the results.

Another possibility that we could not induce infection in this study is that chelonians are not infected orally and other routes of transmission should be investigated. Iridoviruses are variably host specific, and we have shown that a tortoise isolate was capable of creating disease in leopard frogs. Also, we have shown in previous studies that *Ranavirus* infections of chelonians create a systemic disease and viral DNA sequence can be amplified from multiple tissues (Johnson *et al.*, unpublished data). Intracytoplasmic inclusion bodies have also been observed in circulating leukocytes in an infected box turtle in Tennessee (Allender *et al.*, In Press). Thus, it is possible that arthropod-borne vectors, such as mosquitoes or other biting insects, might play a role in transmitting the virus from amphibians to chelonians or from chelonians to other chelonians.

An additional finding of interest in this study was the ability of this virus isolated from a tortoise to create disease in an adult leopard frog. Other studies have shown that adult amphibians are often resistant to infection with Frog Virus 3 (Gantress *et al.*, 2003). One frog experimentally inoculated with a tortoise isolate died naturally seven days post-inoculation with histologic lesions consistent with iridovirus including hepatic necrosis and the presence of intracytoplasmic inclusion bodies. This helps to confirm the possibility that a tortoise virus could be pathogenic to a species of a different class such as an amphibian, and therefore, it is possible that an amphibian virus could be pathogenic to a tortoise. However we cannot rule out that this frog was infected upon arrival. While PCR of oral and cloacal swabs did not amplify viral DNA sequences, the findings of the

full study showed that only 2 of 3 infected frogs had positive PCR on swabs collected ante-mortem. Because we inoculated this frog with virus, we cannot rule out that we super-infected this individual which may have overloaded the immune system resulting in the death of the animal. Along the same lines, this study also provides a rough prevalence of infected amphibians being sold at biological supply companies. About 4% of the frogs ordered from the company were already infected upon arrival, although they appeared clinically healthy during the two weeks they were housed in the facility. This could have significant effects in terms of dispersal of infection if schools or other organizations bought them for a study and then released them into local environments. Additionally, this study has shown that PCR of oral and cloacal swabs can be a useful method for detecting active iridovirus infections of amphibians. While we missed one of the three infected frogs, we were able to amplify sequences in 2 frogs ante-mortem. This could provide useful for detecting the presence of disease in populations with low numbers of individuals where sacrificing adult or young amphibians would be detrimental to the overall success of the population. Prevalence rates of infection however, might be underestimated.

In summary, our study attempted to demonstrate an oral route of transmission of a *Ranavirus* from infected amphibians to chelonians. We tube-fed infected frog homogenates to turtles over six weeks and monitored them for signs of clinical disease and for production of anti-Ranavirus antibodies which might suggest an exposure. We were unable to confirm that chelonians can become infected by ingesting dead amphibians. However, further studies allowing turtles to eat infected amphibians *ad lib* might provide different results as well as altering the conditions of the inoculation such as

environmental temperatures and viral load. Additional methods of transmission should be investigated such as a vector-borne route of transmission. Determining the route of transmission of *Ranavirus* to chelonians will allow for better management and prevention of disease in captive and wild populations.

Table 5-1. Results of the pilot frog study. Day = days post inoculation that frogs were euthanized. PCR = result of the polymerase chain reaction. Histologic change is recorded as positive if lesions were observed consistent with iridovirus infections.

Frog	Infected	Control	Day	PCR	VI	Titer ($TCID_{50}$)	Histologic Change	Inclusion Bodies
1	X		3	+	+	10^4	+	-
2	X		3	-	-	N/A	-	-
3	X		3	-	-	N/A	-	-
4	X		7	+	+	$10^{4.5}$	+	+
5	X		7	+	+	$10^{6.75}$	+	-
6	X		7	+	+	10^4	+	-
7	X		14	+	+	$10^{5.75}$	+	-
8	X		14	-	-	N/A	-	-
9	X		14	-	-	N/A	-	-
10		X	14	-	-	N/A	-	-
11		X	14	-	-	N/A	-	-
12		X	14	-	-	N/A	-	-
13		X	14	-	-	N/A	-	-

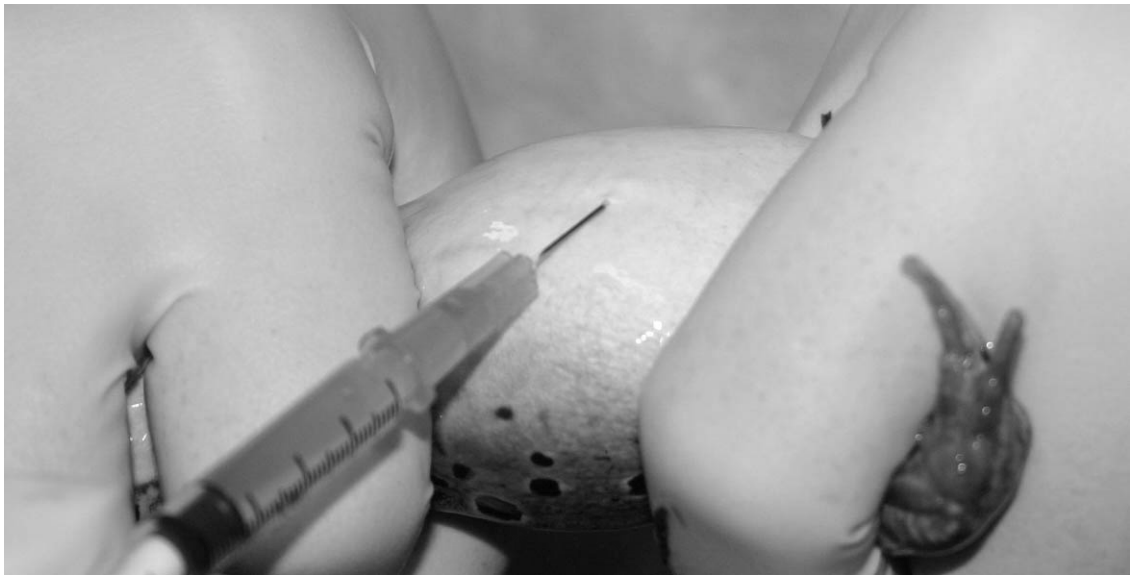


Fig. 5-1. Photograph demonstrating injection of virus infected cell culture media into a ventral lymph sac in a leopard frog (*Rana pipiens*).

Fig. 5-2. Photograph demonstrating the placement of a feeding tube in a red-eared slider (*Trachemys scripta elegans*) for administering frog homogenates directly into the caudal esophagus.

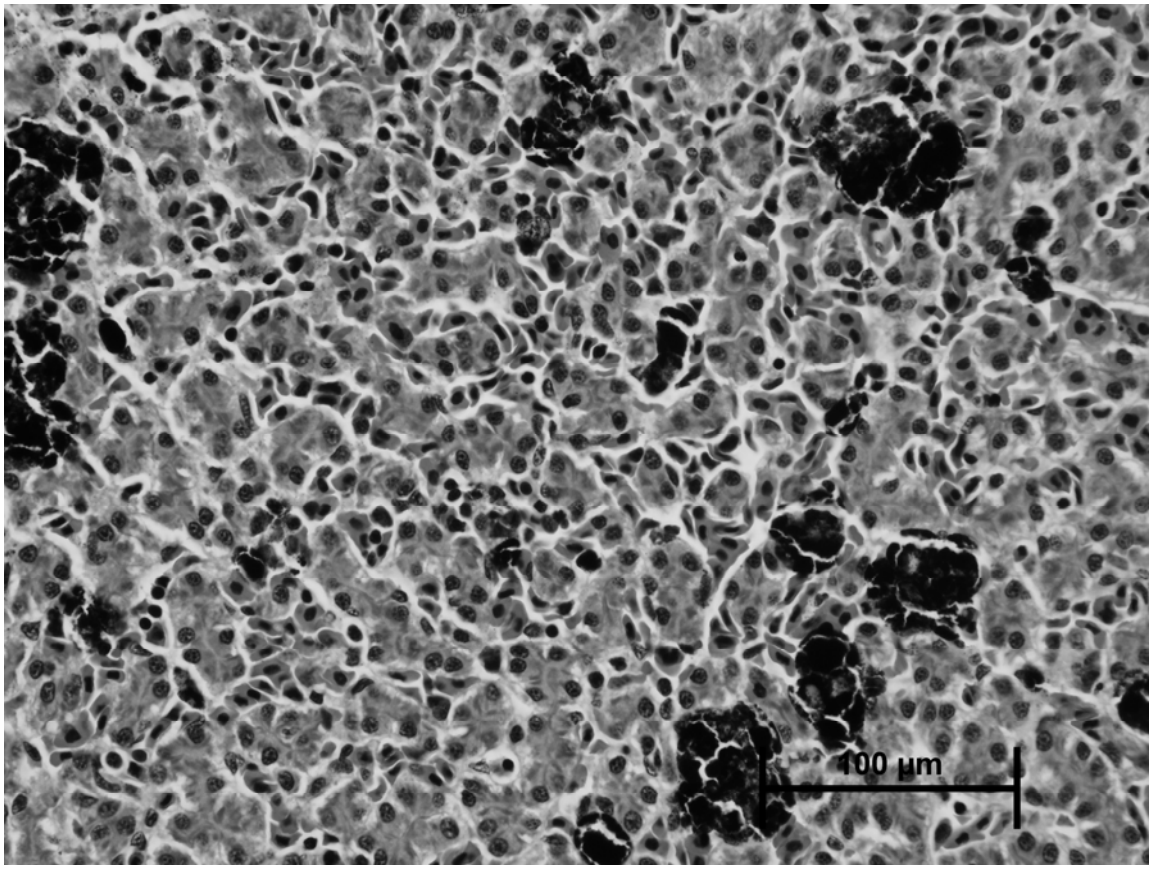


Fig. 5-3. Photomicrograph demonstrating the normal architecture of a liver in a leopard frog (*Rana berlandieri*).

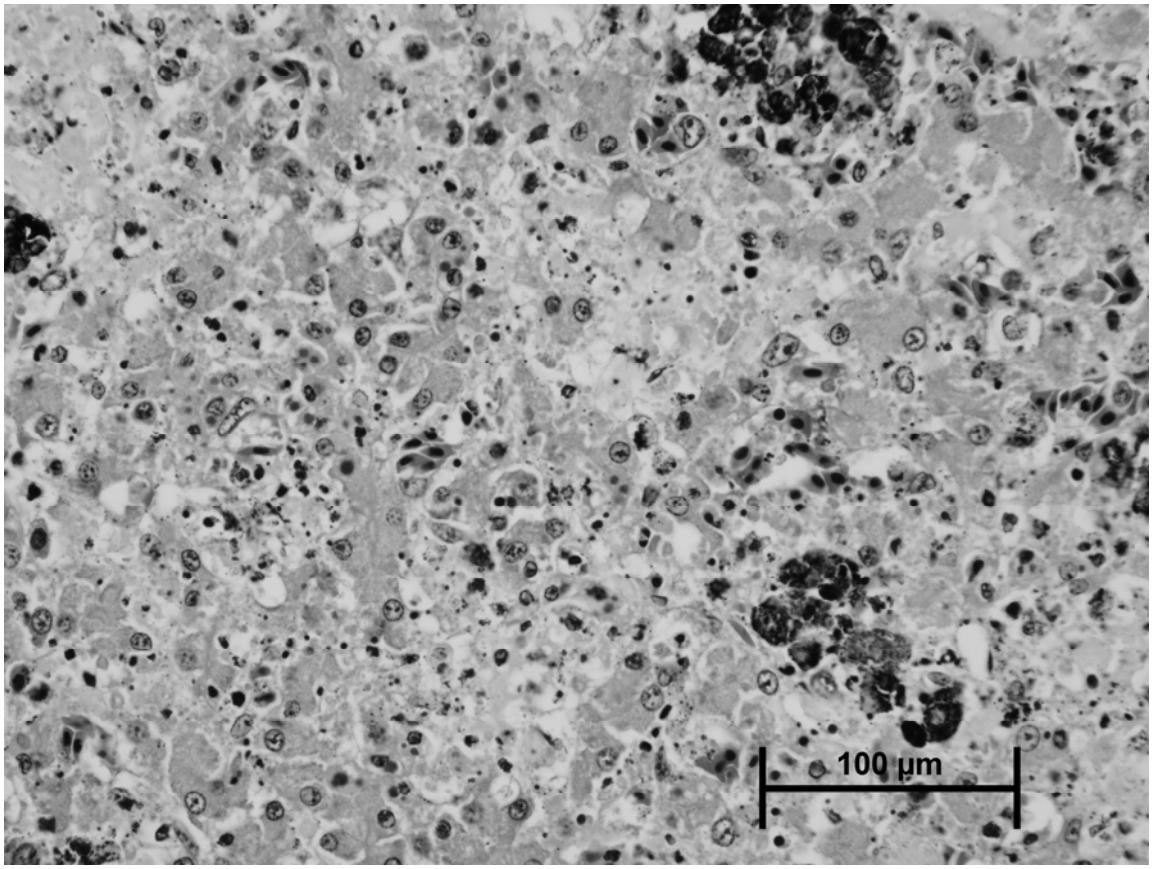


Fig. 5-4. Photomicrograph demonstrating multifocal hepatic necrosis in a leopard frog (*Rana berlandieri*) experimentally inoculated with a *Ranavirus* isolated from a Burmese star tortoise (*Geochelone platynota*).

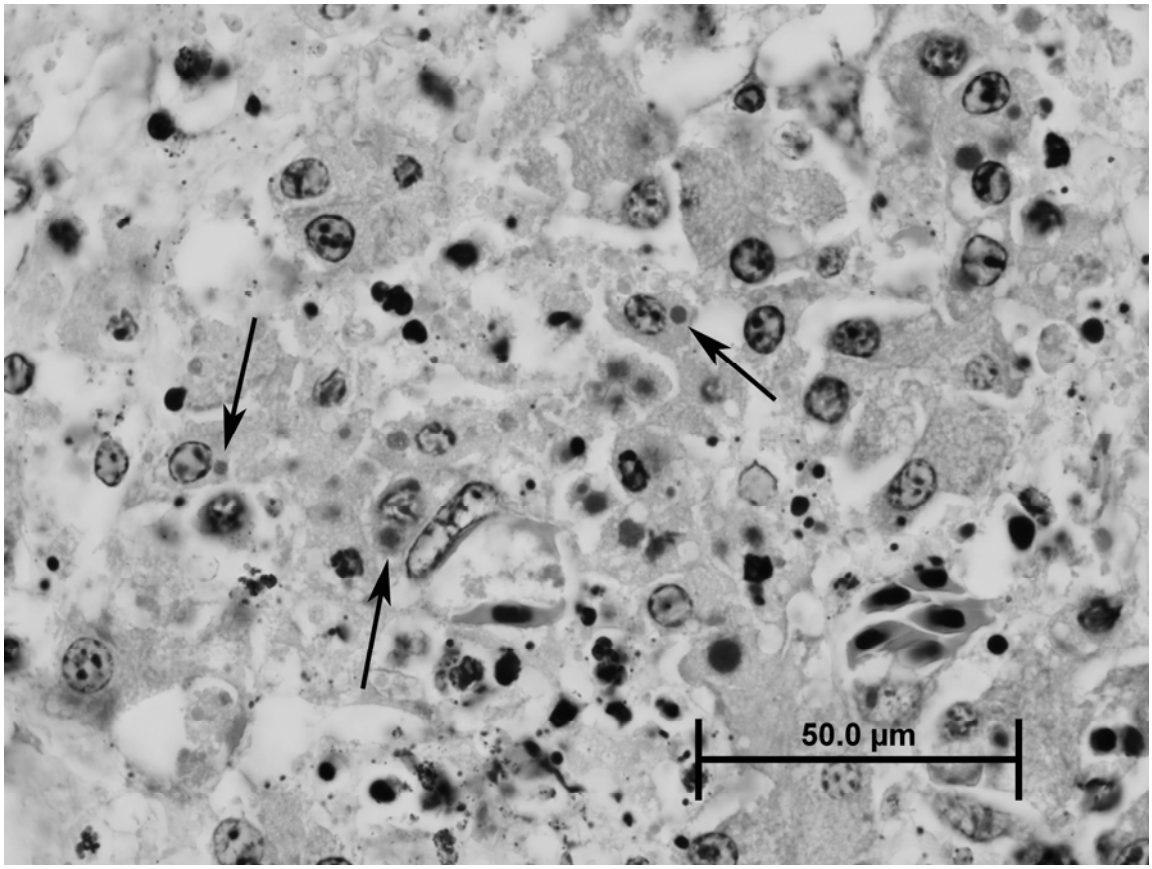


Fig. 5-5. Photomicrograph at higher magnification of the leopard frog (*Rana berlandieri*) liver shown in Fig. 5-4. Arrows denote the presence of intracytoplasmic inclusion bodies consistent with iridovirus infections in amphibians.

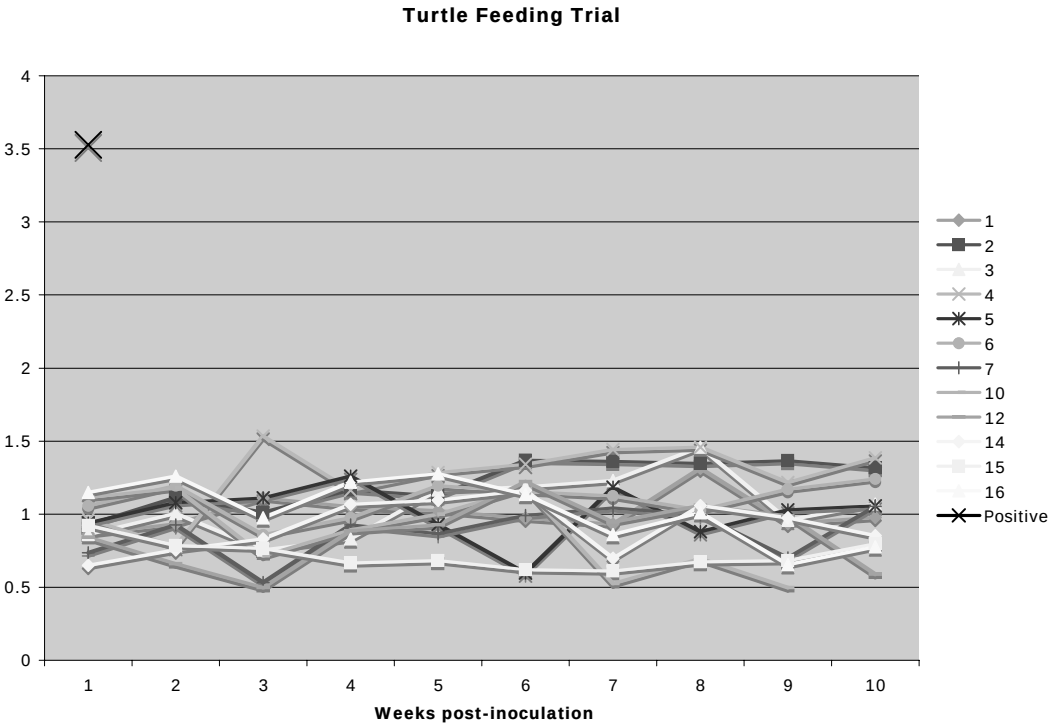


Fig. 5-6. ELISA results graphed as positive to negative (P/N) ratios. Samples were assayed weekly for the duration of the study. A positive control value is included for comparison of values. All turtles remained negative throughout the study.

CHAPTER 6
IN VITRO EFFICACY OF ACYCLOVIR AS A POTENTIAL THERAPEUTIC AGENT FOR IRIDOVIRUS INFECTIONS IN CHELONIANS

Introduction

Iridoviruses have been shown to be pathogenic in chelonians both in the wild and captivity (Marschang *et al.*, 1996; Westhouse *et al.*, 1996; DeVoe *et al.*, 2004). The method of transmission remains unknown, but could be a result of ingesting infected amphibians or exposure to infected water sources. Both have been shown to be a route of transmission in experimental inoculations of salamanders (Jancovich *et al.*, 2001; Pearman *et al.*, 2004). Other routes of transmission such as vector-borne transmission could potentially play a role such as transmission through mosquitoes or other biting insects, although this has not been previously investigated. Regardless of route of infection, chelonians housed in outdoor environments at breeding facilities, in private and zoological collections may be at risk for exposure and development of effective treatment methods would be valuable for captive management of these infections. Previously, treatment of infected chelonians has consisted of supportive care including intracoelomic or subcutaneous fluids, and antibiotics to reduce opportunistic infections. Antiviral therapy has been used to treat herpesvirus infections in tortoises (Marschang *et al.*, 1997), and while pharmacokinetics studies have not yet been performed, the recommended dose for treatment with acyclovir is 80mg/kg orally once daily (Funk and Diethelm, 2006). Marschang *et al.*, (1997) evaluated the in vitro efficacy of two doses of acyclovir and

gancyclovir (25µg/ml and 50µg/ml) at reducing replication of herpesviruses in cell culture and found them to be very effective.

Acyclovir is a guanine analogue antiviral drug. It is closely related to DNA except that it lacks the 2'- and 3' carbons and 3'-hydroxyl group of the deoxyribose ring (Elion, 1993). Acyclovir needs to be phosphorylated three times to acyclovir triphosphate in order to be an active at blocking virus replication. The virus thymidine kinase (TK) enzyme in some herpesviruses can perform the initial phosphorylation of the acyclovir, unlike the cellular TK, which cannot. This prevents acyclovir from stopping replication of cellular DNA, but allows for inhibition of viral DNA replication. A cellular enzyme, guanylate kinase, performs a second phosphorylation (Miller and Miller, 1980). A third phosphorylation then occurs which can be done by a number of cellular enzymes (Elion, 1993). Once phosphorylated three times, the antiviral competes with deoxyguanosine triphosphate (dGTP) for the viral DNA polymerase. Once acyclovir is inserted into the new strand of the replicating DNA chain, replication stops because of the lack of a 3'-hydroxyl group needed to form the phosphodiester bond to the next deoxynucleoside triphosphate (dNTP).

While some species of herpesviruses have TK's that are capable of phosphorylating and thus activating acyclovir, other herpesviruses, such as human cytomegalovirus, are much less sensitive to acyclovir (Elion, 1993). Other large DNA viruses also have TK genes such as poxviruses (Hruby *et al.*, 1983) and African swine fever virus (Blasco *et al.*, 1990). Several isolates of iridoviruses have been shown to have putative thymidine kinase genes including Chilo iridescent virus (Jakob *et al.*, 2001), and grouper iridovirus (Tsai *et al.*, 2005). Functional thymidine kinases have been found in fish lymphocystis

disease virus (Scholz *et al.*, 1988) and Bohle iridovirus (Coupar *et al.*, 2005). Coupar *et al.*, (2005) sequenced and compared homologous open reading frames from epizootic haematopoietic necrosis virus (EHNV) from fish, Wamena iridovirus (WIV) from green pythons and frog virus 3 (FV3). High levels of homology at the nucleotide level were shared among isolates suggesting all to be ORF's encoding thymidine kinase. When compared to other DNA virus TK genes and cellular TK genes, iridoviruses and herpesviruses appeared more closely related to the mitochondrial TKs and to cellular deoxycytidine kinases, whereas poxviruses were more closely related to the cellular TKs (Coupar *et al.*, 2005). This suggests that if iridovirus TK genes are similar to herpesvirus TK genes, then it is plausible that they too could phosphorylate and activate acyclovir.

This study was performed to determine if acyclovir is effective at eliminating cytopathic effect in *Ranavirus* infected cell monolayers, and/or to determine if virus titers were reduced in cell cultures treated with varying concentrations of acyclovir. Results of this study will determine if the antiviral compound, acyclovir, should be considered for use in treating infected chelonians.

Materials and Methods

Cell Cultures

Terrapene heart cells (TH-1) were acquired from the American Type Culture Collection (ATCC-CCL 50; Rockville, MD) and grown to confluency in $2cm^2$ wells of 24 well cell culture plates (BD Biosciences, San Jose, CA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Carlsbad, CA) supplemented with 5% fetal bovine serum (Gibco), gentamicin (60mg/liter; Sigma, St. Louis, MO), penicillin G (120,000 U/liter), streptomycin (120,000 U/liter) and amphotericin B (300µg/liter; Sigma). Cells were grown at 28ºC in the presence of 5% CO_2.

Virus

A *Ranavirus* previously isolated from a Burmese star tortoise (BSTRV; *Geochelone platynota*) was used to determine the efficacy of acyclovir *in vitro*. Briefly, transmission electron microscopy of BSTRV inoculated TH-1 cells showed arrays of virus-like particles in the cytoplasm of infected cells consistent in size and shape with iridoviruses. Polymerase chain reaction targeting a portion of the major capsid protein gene of ranaviruses followed by DNA sequencing demonstrated that the BSTRV isolate shared 100% sequence identity with Frog Virus 3, the type species for the genus *Ranavirus* in the family Iridoviridae. Restriction enzyme digests of BSTRV compared with FV3 showed identical restriction patterns using two enzymes, *Hind*III and *Xba*I, indicating that BSTRV is either identical or closely related to FV3. Intramuscular inoculation of BSTRV into western ornate box turtles (*Terrapene ornata ornata*) and red-eared sliders (*Trachemys scripta elegans*) resulted in high mortality rates.

The BSTRV isolate was titered using a tissue culture infectious dose 50 ($TCID_{50}$) assay from infected media of a third passage isolate. One hundred microliters of the stock virus was diluted in 9.9 mls of cell culture media making a 1:100 dilution and ten fold serial dilutions were made from this dilution six times. One milliliter of each virus dilution was added to 4 wells of a 24 well plate. Cells were incubated for 5 days at 28ºC and then observed for cytopathic effects including cell rounding and detachment from the plate. The $TCID_{50}$ value is the dilution at which 50% of the wells are infected with virus. Virus utilized in this study was determined to be $10^5 TCID_{50}$.

Acyclovir and Concentrations

Acyclovir was purchased from American Pharmaceutical Partners, Inc. (Schaumburg, IL). Acyclovir was diluted into 25mls of the cell culture media described

above at the following concentrations: 0, 0.2, 1, 5, 10, and 25µg/ml. The pH was measured on an aliquot of each to ensure that the pH of the media containing acyclovir did not differ from the pH of the media. Concentrations in media were determined from concentrations found to be effective against herpesviruses, another large double stranded DNA virus, *in vitro* (Kimura *et al.*, 1983; Buck and Loh, 1985; Marschang *et al.*, 1997).

Cytotoxicity Assays

Cytotoxicity can be mistaken for cytopathic effects, so it is important to run a control for this alongside the CPE reduction assays. Four wells of a 24 well culture plate with confluent TH-1 monolayers were incubated with 1ml of media containing acyclovir at one of the following six doses: 0, 0.2, 1, 5, 10 or 25µg/ml. Cell cultures were incubated at 28ºC for four days. After four days, cell cultures were observed for the presence of cytotoxicity.

Cytopathic Effects (CPE) Reduction Assays

Acyclovir was evaluated for its ability to reduce replication of virus by observing for CPE reduction in treated cells. TH-1 cells were grown to confluency in 24 well cell culture plates. Two sets of controls were used. The first control consisted of cells receiving media without virus or acyclovir to make sure the media was not creating any CPE. The second set of controls received virus but no acyclovir to ensure the presence and titer of virus in the media. The latter set of controls and the remainder of the cells received 1ml of virus diluted 1:10 in cell culture media and were incubated at 28ºC for two hours. After two hours, the media was removed, cells were washed once in 1x Hanks balanced salt solution (Gibco) and media containing acyclovir at 0, 0.2, 1, 5, 10 or 25µg/ml was added. Each concentration was done in triplicate. Cells were allowed to incubate for four days, after which, wells were observed for the presence of CPE.

Virus Titer Reduction Assays

The efficacy of acyclovir against iridovirus was also evaluated by virus titer reduction assays. After cell cultures were incubated for four days in the CPE reduction assays, the cell cultures were frozen and thawed three times and the media in the triplicate wells were placed into 15ml centrifuge tubes and centrifuged briefly to remove cell debris. Supernatant from each concentration was then assayed as above for the average $TCID_{50}$ resulting from each concentration including the control, which contained virus without any acyclovir. This indicates the quantitative reduction of virus replication resulting from the presence of acyclovir.

Results

After four days incubation with the various doses of acyclovir, all wells showed CPE at all doses (Table 1). Cytotoxicity or cytopathic effects resulting from the presence of the antiviral compound as opposed to those induced by virus were not observed in any of the wells (Table 1). The virus titer reduction assay showed a slight decrease in titer with increasing dose of acyclovir. Cultures with no or 0.2μg/ml of acyclovir in the cell culture media had a $TCID_{50}$ of $10^{4.8}$/ml. As the dose increased to 1 or 5μg/ml, the titer decreased slightly to $10^{4.5}TCID_{50}$/ml. A further decrease was seen with 10 and 25μg/ml to $10^{4.2}$ and $10^{3.8}TCID_{50}$, respectively (Table 1, Fig. 6-1). While a trend was seen towards a decreasing titer with an increase in concentration of acyclovir, the decrease was not found to be statistically significant ($p=0.287$).

Discussion

This preliminary investigation into a potential treatment for chelonian iridovirus infections demonstrated that acyclovir was not capable of completely inhibiting viral

replication, although it showed a non-statistically significant trend towards decreasing virus titers with increasing concentrations of acyclovir. While this study evaluated the *in vitro* efficacy of acyclovir up to 25μg/ml, higher doses were not evaluated. One study evaluating the efficacy of acyclovir against feline herpesvirus-1 found that 56μg/ml of acyclovir was necessary to reduce plaque numbers to 50% of untreated infections (van der Meulen *et al.*, 2006). Therefore, evaluation of the efficacy of higher doses of acyclovir against iridovirus replication is warranted, but should be evaluated for their harmful potential both *in vitro* and *in vivo* in the species of interest prior to being recommended as a method of treatment. Unpublished reports exist of acyclovir being used successfully at the current recommended dose of 80 mg/kg/day orally (Funk and Diethelm, 2006) to treat iridovirus infected chelonians (T. Norton, R. Ashton, personal communications), in addition to supportive care including fluid therapy and broad-spectrum antibiotics. In both of these instances, the tortoises survived the infection, two have gone on to successfully reproduce, and no apparent harmful side effects were noted. However, it remains unknown if the recovery was in fact due to the acyclovir or to the supportive care.

Pharmacokinetic studies are still needed to determine the bioavailability, elimination half-life, therapeutic dose and dosing intervals for chelonians with different routes of administration. An oral route of administration is usually more desirable than intravenous or intramuscular administration as it is less invasive, and is the currently recommended route of administration for acyclovir use in chelonians (Funk and Diethelm, 2006). Valacyclovir, however, is a pro-drug of acyclovir that has better oral bioavailability, and thus, requires fewer and lower doses to achieve superior plasma

acyclovir levels in humans (MacDougall and Guglielmo, 2004). Valacyclovir doses for human use are about one third of the recommended dose of acyclovir. Pharmacokinetic studies of both drugs would be useful for future administration of acyclovir for both iridovirus and herpesvirus infections.

Another factor that might have affected the results of this study is the timing of acyclovir administration. To mimic a realistic infection, virus was introduced prior to adding media containing acyclovir. Results may have varied if cells were pretreated with acyclovir, if acyclovir was introduced at the time of virus administration or earlier than 2 hours post adsorption of virus to cell monolayers. A study looking at the efficacy of acyclovir in reducing the replication of channel catfish herpesvirus *in vitro* showed that the earlier the antiviral drug is added, the more virus replication is suppressed (Buck and Loh, 1985). While acyclovir added at 0 and 1.5 hours after adsorption of virus to cell cultures resulted in 81-99% inhibition of herpesvirus replication, no inhibition was seen when acyclovir was added 5 hours after adsorption. Thus, further studies could determine if addition of acyclovir earlier than 2 hours post adsorption might result in a further increase in inhibition of iridovirus replication.

While acyclovir may or may not be helpful in reducing *in vivo* virus replication of iridovirus, other drugs should similarly be evaluated for their ability to inhibit iridovirus replication. Acylcovir was chosen for this study based on the similarity of the iridovirus TK genes to herpesvirus TK genes, and the successful use of acyclovir to treat human and other herpesvirus infections. Other nucleoside analogue antiviral drugs should be evaluated such as gancyclovir and vidarabine.

This study has shown that there is a non-statistically significant trend in decreasing virus titers with increasing doses of acyclovir in *Ranavirus* infected cell culture monolayers. This represents the first investigation into an antiviral drug as a potential treatment for iridovirus infected chelonians.

Table 6-1. Effect of increasing doses of acyclovir on cytopathic effect reduction, cytotoxicity and $TCID_{50}$ of *Terrapene* heart cells inoculated with BSTRV.

Acyclovir Concentration (μg/ml)	No. of wells showing CPE (n=3)	No. of wells showing cytotoxicity (n=4)	Virus titer ($TCID_{50}$)
0	3	0	$10^{4.8}$
0.2	3	0	$10^{4.8}$
1	3	0	$10^{4.5}$
5	3	0	$10^{4.5}$
10	3	0	$10^{4.2}$
25	3	0	$10^{3.8}$

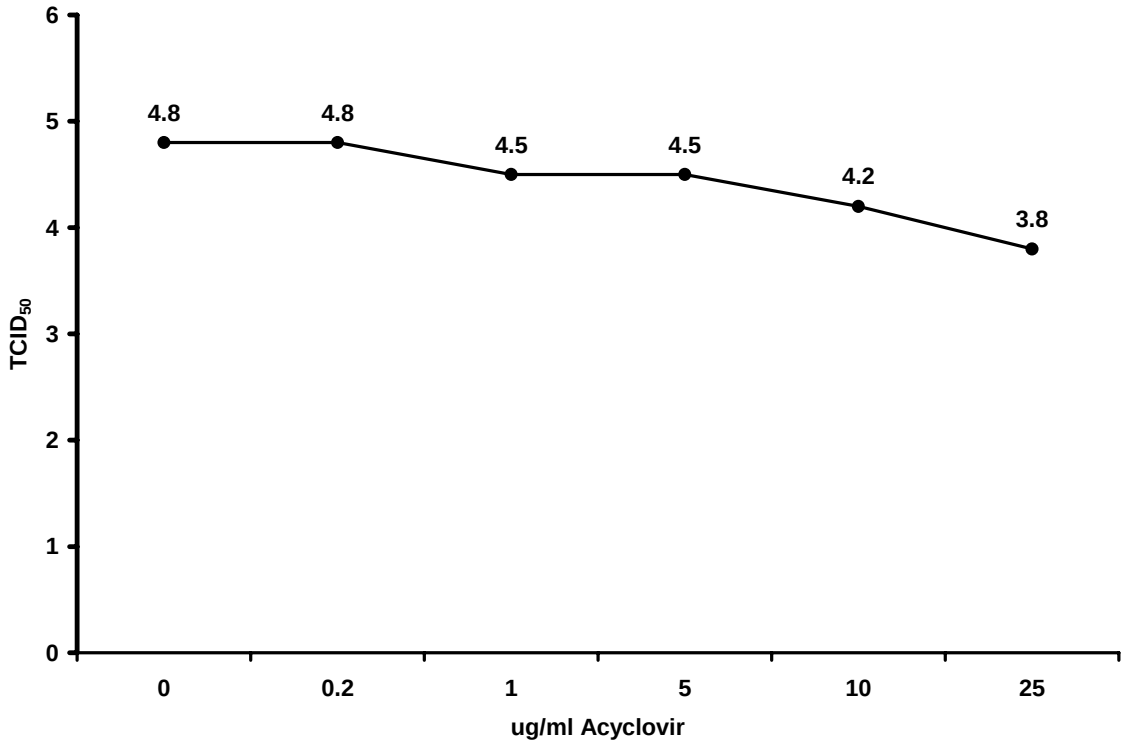


Fig. 6-1. Effect of increasing concentrations of acyclovir on the $TCID_{50}$ of *Terrapene* cells inoculated with the Burmese star tortoise isolate.

CHAPTER 7
CONCLUSIONS AND FUTURE RESEARCH

It has been suggested that chelonians face a more serious threat of decline than that posed by the well-publicized decline of amphibian populations (Klemens, 2000). Two thirds of all species of freshwater turtles and tortoises are currently listed as threatened on the IUCN Red List of Threatened Species (Turtle Conservation Fund, 2002). Chelonians have low fecundity, low juvenile survival rate, and a long adult lifespan; a life history strategy where loss of adult animals (such as loss by disease) has a significant impact on population recovery (Heppell, 1998). Emerging infectious diseases have been increasingly recognized as factors influencing wildlife health and populations (Harvell *et al*., 1999; Daszak *et al*., 2000) and the cause(s) of mass mortality events in wild chelonian populations often remain undetermined (Flanagan, 2000; Dodd, 2001).

This study fulfills Koch's postulates, conclusively identifying *Ranavirus* as a causative agent of mortality in chelonians. It provides a comprehensive review of histologic changes observed in both naturally and experimentally infected chelonians, which will prove useful to pathologists, wildlife veterinarians, and biologists in the future. Review of archived materials suggests that chelonian iridovirus infections date back to at least 1991 and recent and historic cases have defined a much larger geographic range of prevalence than previously known, spanning from Texas to New York.

The seroprevalence rate of exposure in free-ranging gopher tortoises was determined at 1.5%, although we suspect that this is an underestimate of the true exposure rate in natural settings: an artifact of the severity and short duration of disease

as determined by experimental transmission studies. The seroprevalence of free-ranging box turtles was not evaluated. Box turtles are more carnivorous than gopher tortoises, so if ingestion of amphibians is a major source of infection, we might expect to see a higher rate of prevalence in box turtles than in gopher tortoises. Similarly, fresh water turtles should be evaluated for their rates of exposure, because they are likely in more direct contact with virus particles in infected bodies of water. Further evaluation of species susceptibility should also be performed. While three of five Burmese star tortoises became ill, of which one died, several other species of tortoises were in adjacent pens and remained clinically healthy, suggesting that some susceptibility differences exist between species.

Persistent infections should also be evaluated further. This is known to occur in amphibians, and result of one transmission study showed that one turtle remaining clinically healthy was shedding virus 30 days post-inoculation. Studies with a larger sample size and longer duration would help to confirm this finding. Also determining whether or not the virus being shed was infectious would be helpful in determining how to handle recovering individuals within managed populations.

Another study that should be focused on in the future is confirmation that chelonians can become infected with amphibian viruses. While we were able to show that a tortoise isolate was capable of infecting other turtles, and that this virus appeared to be closely related to, if not the same strain as the frog isolate, we could not be 100% sure that this virus came from an amphibian. We did however show that the tortoise isolate was able to create disease in at least one frog, with histologic confirmation of lesions

consistent with iridovirus infection. A further study taking a frog isolate and inoculating turtles would help to confirm that amphibians could serve as a source of infection.

The most important question this study failed to answer was how this virus is being transmitted. We hypothesized that amphibians may be a source of infection, as isolates found in amphibians at the site of two turtle deaths had 100% shared sequence identity across a portion of the major capsid protein gene with the turtle isolates. Restriction enzyme analysis of one tortoise and frog isolate showed identical patterns suggesting that they are infected with the same strain or closely related strains of virus. If amphibians really are the source of infection, than chelonians worldwide are at risk of exposure in areas where they overlap with amphibian populations. Chelonians at the site where the frog isolate was obtained were observed on two occasions ingesting dead amphibians, and thus we suspected that transmission might occur by way of an oral route. However, we were unable to replicate an oral route of transmission, both using a single concentrated dose of virus in cell culture media as well as by repeated dosing of turtles with infected frogs. Further studies should focus on identifying the route of transmission, as this is a critical piece of information in managing diseased populations or in preventing disease. Vector-borne routes of infection should really be considered because this virus causes systemic infections.

In summary, this study has shown that iridoviruses in the genus *Ranavirus* can be highly pathogenic to turtles and tortoises. They may be a significant cause of mortality in wild populations, and die-offs would likely be difficult to detect based on the duration of disease. While we were able to provide some answers regarding the pathogenesis of this

virus in chelonians, there are many questions that remain unanswered and to which future studies should be directed.

LIST OF REFERENCES

Allender, M.C., M.M. Fry, A.R. Irizarry, L. Craig, A.J. Johnson, and M. Jones. In press. Intracytoplasmic inclusions in circulating leukocytes from an eastern box turtle (*Terrapene carolina carolina*) with iridoviral infection. J. Wildl. Dis.

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acid Res. **25**:3389-3402.

Ariel, E. 1997. Pathology and serological aspects of Bohle iridovirus infections in six selected water-associated reptiles in North Queensland. PhD thesis. James Cook University, Queensland.

Blasco, R., C. Lopez-Otin, M. Munoz, E.O. Bockamp, C. Simon-Mateio, E. Vinuela. 1990. Sequence and evolutionary relationships of African swine fever virus thymidine kinase. Virol. **178**:301-304.

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

Bradford, D.F. 1991. Mass mortality and extincition in a high-elevation population of *Rana muscosa*. J. Herpetol. **25**:174-177.

Brown, M.B., G.S. McLaughlin, P.A. Klein, B.C. Crenshaw, I.M. Schumacher, D.R. Brown, and E.R. Jacobson. 1999. Upper respiratory tract disease in the gopher tortoise is caused by *Mycoplasma agassizii*. J. Clin. Microbiol. **37**:2262-2269.

Brown, D.R., I.M. Schumacher, G.S. McLaughlin, M.B. Brown, P.A. Klein, and E.R. Jacobson. 2002. Application of diagnostic tests for mycoplasmal infections of desert and gopher tortoises, with management recommendations. Chelon. Conserv. Biol. **4**:497-507.

Brown, D.R., I.M. Schumacher, M.F. Nogueira, L.J. Richey, L.A. Zacher, T.R. Schoeb, K.A. Vliet, R.A. Bennett, E.R. Jacobson and M.B. Brown. 2001. Detection of antibodies to a pathogenic mycoplasma in American alligators (*Alligator mississippiensis*), broad-nosed Caimans (*Caiman latirostris*), and Siamese crocodiles (*Crocodylus siamensis*). J. Clin. Microbiol. **39**:285-292.

Brown, J.D., and J.M. Sleeman. 2002. Morbidity and mortality of reptiles admitted to the Wildlife Center of Virginia, 1991 to 2000. J. Wildl. Dis. **38**:699-705.

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

Brunner, J.L., D.M. Schock, E.W. Davidson, and J.P. Collins. 2004. Intraspecific reservoirs: complex life history and the persistence of a lethal ranavirus. Ecology. **85**:560-566.

Buck, C.C., and P.C. Loh. 1985. In vitro effect of acyclovir and other antiherpetic compounds on the replication of channel catfish virus. Antiviral Res. **5**:269-280.

Burkhardt, E., H. Müller. 1987. Susceptibility of checken blood lymphoblasts and monocytes to infectious bursal disease virus (IBDV). Arch. Virol. **94**:297-303.

Caipang, C.M.A., T. Takano, I. Hirono and T. Aoki. 2006. Genetic vaccines protect red seabream, *Pagrus major*, upon challenge with red seabream iridovirus (RSIV). Fish Shellfish Immunol. **2**:130-138.

Chen, Z., J. Zheng, and Y. Jian. 1999. A new iridovirus isolated from soft-shelled turtle. Virus Res. **63**:147-151.

Chinchar, V.G. 2002. Ranaviruses (family *Iridoviridae*): emerging cold-blooded killers. Brief review. Arch. Virol. **147**:447-470.

Chinchar, V.G., J. Wang, G. Murti, C. Carey, and L. Rollins-Smith. 2001. Inactivation of frog virus 3 and channel catfish virus by esculentin-2P and ranatuerin-2P, two antimicrobial peptides isolated from frog skin. Virol. **288**:351-357.

Clark, J.F., G. Gray, F. Fabian, R.F. Ziegel, and D.T. Karzon. 1969. Comparative studies of amphibian cytoplasmic virus strains isolated from the leopard frog, bullfrog, and newt, p. 310-326. *In*: A. Mizell (ed.), Biology of amphibian tumors. Recent results in cancer research. Springer-Verlag, New York.

Cooper, E.L., A.E. Klumpau, and A.G. Zabata. 1985. Chapter 8: Reptilian immunity, p. 599-678. *In:* C. Gans (ed.), Biology of the Reptilia, Vol 14, Morphology. Academic Press, New York.

Coupar, B.E.H., S.G. Goldie, A.D. Hyatt and J.A. Pallister. 2005. Identification of a Bohle iridovirus thymidine kinase gene and demonstration of activity using vaccinia virus. Arch. Virol. **150**:1797-1812.

Crowther, J.R. 2001. The ELISA guidebook, p. 301-345. Humana Press, Totowa, New Jersey.

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

Cunningham, A.A., T.E.S. Langton, P.M. Bennett, J.F. Lewin, S.E.N. Drury, R.E. Gough, and S.K. Macgregor. 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Philos. Trans. R. Lond. B Biol. Sci. **351**:1529-1557.

Daszak, P., L. Berger, A.A. Cunningham, A.D. Hyatt, D.E. Green and R. Speare. 1999. Emerging infectious diseases and amphibian population declines. Emerg. Infect. Dis. **5**:735-748.

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

DeVoe, R., K. Geissler, K., S. Elmore, D. Rotstein, G. Lewbart and J. Guy. 2004. Ranavirus-associated morbidity and mortality in a group of captive eastern box turtles (*Terrapene carolina carolina*). J. Zoo Wildl. Med. **35**:524-543.

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

Dodd, K.C. 2001. North American box turtles: a natural history. University of Oklahoma Press. Norman, Oklahoma.

Drury, S.E.N., R.E. Gough, and I. Calvert. 2002. Detection and isolation of an iridovirus from chameleons (*Chameleo quadricornis* and *Chameleo hoehnelli*) in the United Kingdom. Vet. Rec. **150**:451-452.

Elion, G.B. 1993. Acyclovir: discovery, mechanism of action and selectivity. J. Med. Virol. Suppl. **1**:2-6.

Evans, A.S. 1976. Causation and disease: the Henle-Koch postulates revisited. Yale J. Biol. Med. **49**:175-195.

Fellers, G.M., and C.A. Drost. 1993. Disappearance of the cascades frog *Rana cascadae* at the southern end of its range, California, USA. Biol. Conserv. **65**:177-181.

Fisher, R.N. and H.B. Shaffer. 1996. The decline of amphibians in California's Great Central Valley. Conserv. Biol. **10**:1387-1397.

Flanagan, J. 2000. Disease and health considerations, p. 85-95. *In:* M.W. Klemens (ed.), Turtle conservation, Smithsonian Institution Press, Washington.

Forson, D., and A. Storfer. 2006. Effects of atrazine and iridovirus infection on survival and life-history traits of the long-toed salamander (*Ambystoma macrodactylum*). Environ. Toxicol. Chem. **25**:168-173.

Funk, R.S., and G. Diethelm. 2006. Reptile formulary, p. 1119-1139. *In:* D.M. Mader (ed.), Reptile Medicine and Surgery, 2nd edition, WB Saunders, Philadelphia.

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

Goorha, R. and K.G. Murti. 1982. The genome of frog virus 3, an animal DNA virus, is circularly permuted and terminally redundant. Proc. Natl. Acad. Sci. U.S.A. **79**:248-252.

Goorha, R., G. Murti, A. Granoff, and R. Tirey. 1978. Macromolecular synthesis in cells infected by frog virus 3. VIII. The nucleus is a site of frog virus 3 DNA and RNA synthesis. Virol. **82**:34-52.

Granoff, A., P.E. Came, and K.A. Rafferty, Jr. 1965. The isolation and properties of viruses from *Rana pipiens*: Their possible relationship to the renal adenocarcinoma of the leopard frog. Ann. N.Y. Acad. Sci. **126**:237-255.

Granoff A., P.E. Came, and D.C. Breeze. 1966. Viruses and renal adenocarcinoma of *Rana pipiens*. I. The isolation and properties of virus from normal and tumor tissue. Virol. **29**:133-148.

Green, D.E., K.A. Converse, and A.K. Schrader. 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996-2001. Ann. N.Y. Acad. Sci. **969**:323-339.

Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Harvell, C.D., K. Kim, J.M. Burkholder, R.R. Colwell, P.R. Epstein, D.J. Grimes, E.E. Hofmann, E.K. Lipp, A.D. Osterhaus, R.M. Overstreet, J.W. Porter, G.W. Smith, and G.R. Vasta. 1999. Emerging marine diseases—climate links and anthropogenic factors. Science. **285**:1505-1510.

Heldstab, A, and G. Bestetti. 1982. Spontaneous viral hepatitis in a spur-tailed Mediterranean land tortoise (*Testudo hermanni*). J. Zoo Anim. Med. **13**:113-120.

Heppell, S.S. 1998. Application of life-history theory and population model analysis to turtle conservation. Copeia. **2**:367-375.

Herbst, L. and E.R. Jacobson. 1995. Diseases of marine turtles, p. 593-595. *In*: K. Bjorndal (ed.), Biology and Conservation of Sea Turtles. Smithsonian Institution Press, Washington, D.C.

Hruby, D.E., R.A. Maki, D.B. Miller, and L.A. Ball. 1983. Fine structure analysis and nucleotide sequence of the vaccinia virus thymidine kinase gene. Proc. Natl. Acad. Sci. USA. **80**:3411-3415.

Hyatt A.D., M. Williamson, B.E. Coupar, D. Middleton, S.G. Hengstberger, A.R. Gould, P. Selleck, T.G. Wise, J. Kattenbelt, A.A. Cunningham, and J. Lee. 2002. First identification of a ranavirus from green pythons (*Chondropython viridis*). J. Wildl. Dis. **38**:239-252.

Jacobson E.R., J.L. Behler, and J.L. Jarchow. 1999. Health assessment of chelonians and release into the wild, p. 232-242. *In:* M.E. Fowler and R.E. Miller (eds.), Zoo and Wild Animal Medicine, Current Therapy 4, W.B. Saunders Co., Philadelphia.

Jacobson, E.R., A.J. Johnson, J.A. Hernandez, S.J. Tucker, A.P. DuPuis II, R. Stevens, D. Carbonneau, and L. Stark. 2005. Validation and use of an indirect enzyme-linked immunosorbent assay for detection of antibodies to west Nile virus in American alligators (*Alligator mississippiensis*) in Florida. J. Wildl. Dis. **41**:107-114.

Jakob, N.J., K. Muller, U. Bahr, and G. Darai. 2001. Analysis of the first complete DNA sequence of an invertebrate iridovirus: coding strategy of the genome of Chilo iridescent virus. Virol. **286**:182-196.

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

Jancovich, J.K., E.W. Davidson, J.F. Morado, B.L. Jacobs, and J.P. Collins. 2001. Transmission of the *Ambystoma tigrinum* virus to alternative hosts. Dis. Aquat. Org. **46**:159-163.

Johnson, A.J., J.F.X. Wellehan, A.P. Pessier, T.M. Norton, W.R. Belzer, J.W. Brooks, R. Wagner, N.L. Stedman, J. Spratt, and E.R. Jacobson. 2004. Iridovirus infections of turtles and tortoises. Proceedings of the Wildlife Disease Association.

Johnson, A.J., A.P., Pessier, J.F.X. Wellehan, R. Brown, and E.R. Jacobson. 2005. Identification of a novel tortoise herpesvirus from a California desert tortoise (*Gopherus agassizii*). Vet. Microbiol. **111**:107-116.

Johnsrude, J.D., R.E. Raskin, A.Y. Hoge, and G.W. Erdos. 1997. Intraerythrocytic inclusions associated with iridoviral infection in a fer de lance (*Bothrops moojeni*) snake. Vet. Pathol. **34**:235-238.

Just, F., S. Essbauer, W. Ahne, and S. Blahak. 2001. Occurrence of an invertebrate iridescent-like virus (*Iridoviridae*) in reptiles. J. Vet. Med. B Infect. Dis. Vet. Public Health. **48**:685-694.

Kimura, T., S. Suzuki, and M. Yoshimizu. 1983. In vitro antiviral effect of 9-(2-hydroxyethoxymethyl) guanine on the fish herpesvirus, Oncorhynchus masou virus (OMV). Antiviral Res. **3**:93-101.

Klemens, M.W. 2000. Turtle Conservation. Smithsonian Institution Press, Washington.

Kroese, F.G., and N. Van Rooijen. 1982. The architecture of the spleen of the red-eared slider, Chrysemys scripta elegans (Reptilia, Testudines). J. Morphol. **173:**279-284.

Langdon, J.S. 1989. Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis L.*, and 11 other teleosts. J. Fish Dis. **12**:295-310.

Langdon, J.S. and J.D. Humphrey. 1987. Epizootic haematopoietic necrosis, a new viral disease in redfin perch, *Perca fluviatilis* L., in Australia. J. Fish Dis. **10**:289-297.

Laurance, W.F., K.R. McDonald, and R. Speare. 1996. Epidemic disease and the catastrophic decline of Australian rain forest frogs. Conserv. Biol. **10**:406-413.

Lips, K.R. 1999. Mass mortality and population declines of anurans at an upland site in Western Panama. Conserv. Biol. **13**:117-125.

Lopez, M., J.C. Rojas, R. Vandame, and T. Williams. 2002. Parasitoid-mediated transmission of an iridescent virus. J. Invertebr. Pathol. **80**:160-170.

Lorbacher de Ruiz, H. 1990. Hepatotoxicity of iridoviruses, p. 235-245. *In:* G. Darai (ed.), Molecular biology of iridoviruses, Kluwer Academic Publishers, Boston.

MacDougall, C., and B.J. Guglielmo. 2004. Pharmacokinetics of valaciclovir. J. Antimicrob. Chemo. **53**:899-901.

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

Mao, J., D.E. Green, G. Fellers, and V.G. Chinchar. 1999. Molecular characterization of iridoviruses isolated from sympatric amphibians and fish. Virus Res. **63**:45-52.

Mao, J., R.P. Hedrick, and V.G. Chinchar. 1997. Molecular characterization, sequence analysis and taxonomic position of newly isolated fish iridoviruses. Virol. **229**:212-220.

Marschang, R.E., P. Becher, H. Posthaus, P. Wild, H-J. Thiel, U. Müller-Doblies, E.F. Kaleta, and L.N. Bacciarini. 1999. Isolation and characterization of an iridovirus from Hermann's tortoises (*Testudo hermanni*). Arch. Virol. **144**:1909-1922.

Marschang, R.E., S. Braun, P. Becher. 2005. Isolation of a ranavirus from a gecko (*Uroplatus fimbriatus*). J. Zoo Wildl. Med. **36**:295-300.

Marschang, R.E., M. Gravendyck, and E.F. Kaleta. 1997. Herpesvirus in tortoises: investigations into virus isolation and the treatment of viral stomatitis in *Testudo hermanni* and *T. graeca*. J. Vet. Med. **44**:385-394.

McDowell, E.M., and B.F. Trump. 1976. Historical fixatives suitable for diagnostic light and electron microscopy. Arch. Pathol. Lab. Med. **100**:405-414.

Miller, W.H. and R.L. Miller. 1980. Phosphorylation of acyclovir (acycloguanosine) monophosphate by GMP kinase. J. Biol. Chem. **255**:7204-7207.

Moody, N.J.G., and L. Owens. 1994. Experimental demonstration of the pathogenicity of a frog virus, Bohle iridovirus, for a fish species barramundi *Lates calcarifer*. Dis. Aquat. Org. **18**:95-102.

Morse, S.S. 1995. Factors in the emergence of infectious diseases. Emerg. Infect. Dis. **1**:7-15.

Muller, M., N. Zangger, and T. Denzler. 1988. Iridovirus-epidemie bei der griechischen Landschildkrote (*Testudo hermanni hermanni*), p.271-274. Verhandl Ber 30. Int Symp Erkr Zoo-und Wildtiere, Sofia.

Nakajima, K., Y. Maeno, J. Kurita and Y. Inui. 1997. Vaccination against red sea bream iridoviral disease in red sea bream. Fish Pathol. **32**:205-209.

Nakajima, K., Y. Maeno, A. Honda, K. Yokoyama, K. Tooriyama, and S. Manabe. 1999. Effectiveness of a vaccine against red sea bream iridoviral disease in a field trial test. Dis. Aquat. Org. **36**:73-75.

Origgi, F.C., P.A. Klein, K. Mathes, S. Blahak, R.E. Marschang, S.J. Tucker and E.R. Jacobson. 2001. Enzyme-linked immunosorbent assay for detecting

herpesvirus exposure in Mediterranean tortoises (spur-thighed tortoise [*Testudo graeca*] and Hermann's tortoise [*Testudo hermanni*]). J. Clin. Microbiol. **39**:3156-3163.

Orrigi, F.C., C.H. Romero, D.C. Bloom, P.A. Klein, J.M. Gaskin, S.J. Tucker, and E.R. Jacobson. 2004. Experimental transmission of a herpesvirus in Greek tortoises (*Testudo graeca*). Vet. Pathol. **41**:50-61.

Pearman, P.B., T.W. Garner, M. Straub, and U.F. Greber. 2004. Response of the Italian agile frog (*Rana latastei*) to a Ranavirus, frog virus 3: a model for viral emergence in naive populations. J. Wildl. Dis. **40**:660-669.

Reddacliff, L.A., and R.J. Whittington. 1996. Pathology of epizzotic haematopoietic necrosis virus (EHNV) infection in rainbow trout (*Oncorhynchus mykiss Walbaum*) and redfin perch (*Perca fluviatilis L*). J. Comp. Pathol. **115**:103-115.

Rojas, S., K. Richards, J.K. Jancovich, and E.W. Davidson. 2005. Influence of termperature on *Ranavirus* infection in larval salamanders *Ambystoma tigrinum*. Dis. Aquat. Organ. **63**:95-100.

Rossell, C. R., I.M. Rossell, M.M. Orroca, and J.W. Petranka. 2002. Epizootic disease and high mortality in a population of eastern box turtles. Herp Rev. **33**:99-101.

Scholz, J., A. Rosen-Wolff, M. Touray, P. Schnitzler and G. Darai. 1988. Identification, mapping and cloning of the thymidine kinase gene of fish lymphocystis disease virus. Virus Res. **9**:63-72.

Schramm, B. and J.K. Locker. 2005. Cytoplasmic organization of poxvirus DNA replication. Traffic. **6**:839-846.

Schumacher, I.M., M.B. Brown, E.R. Jacobson, B.R. Collins, and P.A. Klein. 1993. Detection of antibodies to a pathogenic mycoplasma in desert tortoises (*Gopherus agassizii*) with upper respiratory tract disease. J. Clin. Micro. **31**:1454-1460.

Seigel, R.A., R.B. Smith, and N.A. Seigel. 2003. Swine flu or 1918 pandemic? Upper respiratory tract disease and sudden mortality of gopher tortoises (*Gopherus polyphemus*) on a protected habitat in Florida. J. Herpetol. **37**:137-144.

Sorby, R., T.N. Wien, G. Husby, A. Espenes, and T. Landsverk. 2005. Filter function and immune complex trapping in splenic ellipsoids. J. Comp. Pathol. **132**:313-321.

Speare, R. and J.R. Smith. 1992. An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. Dis. Aquat. Org. **14**:51-57.

Steiner, K.A., R.J. Whittington, R.K. Petersen, C. Hornitzky,, and H. Garnett. 1991. Purification of epizootic haematopoietic necrosis virus and its detection using ELISA. J. Virol. Meth. **33**:199-209.

Telford, S.R. and E.R. Jacobson. 1993. Lizard erythrocytic virus in east African chameleons. J. Wildl. Dis. **29**:57-63.

Tidona, C.A., P. Schnitzler, R. Kehm, and G. Darai. 1998. Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? Virus Genes. **16**:59-66.

Tokuda, Y., T. Nakanure, K. Satonaka, S. Maeda, K. Doi, S. Baba, and T. Sugiyama. 1990. Fundamental study on the mechanisms of DNA degradation in tissues fixed in formaldehyde. J. Clin. Pathol. **43**:748-751.

Tsai, C-T., J-W. Ting, M-H. Wu, M-W. Wu, I-C. Guo, and C-Y. Chang. 2005. Complete genome sequence of the grouper iridovirus and comparison of genomic organization with those of other iridoviruses. J. Virol. **79**:2010-2023.

Turtle Conservation Fund. 2002. A global action plan for conservation of tortoises and freshwater turtles. Strategy and funding prospectus 2002-2007. M.T.C. Printing, Inc., Leominster, Massachusetts.

United States Fish and Wildlife Service. 1994. Desert tortoise (Mojave population) recovery plan. U.S. Fish and Wildlife Service. Portland, Oregon.

Van der Meulen, K., B. Garre, S. Croubels, and H. Nauwynck. 2006. In vitro comparison of antiviral drugs against feline herpesvirus 1. BMC Vet Res. **26**:13.

VanDevanter, D.R. P. Warrener, L. Bennett, E.R. Schultz, S. Coulter, R.L. Garber, and T.M. Rose. 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. J. Clin. Microbiol. **34**:1666-1671.

Warr, G.W., Magor, K.E., and D.A. Higgins. 1995. IgY: clues to the origins of modern antibodies. Immunol. Today. **16**:392-398.

Westhouse, R.A., E.R. Jacobson, R.K. Harris, K.R. Winter, and B.L. Homer. 1996. Respiratory and pharyngo-esophageal iridovirus infection in a gopher tortoise (*Gopherus polyphemus*). J. Wildl. Dis. **32**:682-686.

Whittington, R.J., C. Kearns, and R. Speare. 1997. Detection of antibodies against iridoviruses in the serum of the amphibian *Bufo marinus*. J. Virol Meth. **68**:105-108.

Williams, R.C., and K.M. Smith. 1957. A cristallizable insect virus. Nature. **179**:119-120.

Williams, T. 1996. The iridoviruses. Adv. Virus Res. **46**:345-412.

Williams, T., V. Barbosa-Solomieu, and V.G. Chinchar. 2005. A decade of advances in iridovirus research. Adv. Virus Res. **65**:173-248.

Wolf, K., G.L. Bullock, C.E. Dunbar, and M.C. Quimby. 1968. Tadpole edema virus: a viscerotrophic pathogen for anuran amphibians. J. Infect. Dis. **118**:253-262.

Xeros, N. 1964. A second virus disease of the leather jacket, *Tipula paludosa*. Nature. **174**:562-563.

Zupanovic, Z., G. Lopez, A.D. Hyatt, B. Green, G. Bartran, H. Parkes, R.J. Whittington, and R. Speare. 1998. Giant toads Bufo marinus in Australia and Venezuela have antibodies against 'ranaviruses'. Dis. Aquat. Organ. **32**:1-8.

BIOGRAPHICAL SKETCH

April Johnson received a Bachelor of Science in biology from Cedarville University in July 1998. She then attended the University of Illinois, College of Veterinary Medicine, where she received a Doctor of Veterinary Medicine in May 2002. She enrolled in the PhD program at the University of Florida, College of Veterinary Medicine, during the fall of that same year. She concurrently enrolled in the Master of Public Health program with an emphasis in epidemiology at the University of Florida, College of Public Health and Health Professions, in the fall of 2003, which she completed in the spring of 2006. In July of 2006, she started a two-year fellowship as an Epidemic Intelligence Service officer with the Commissioned Corps of the U.S. Public Health Service at the Centers for Disease Control and Prevention in Atlanta, Georgia, where she will focus on the epidemiology and prevention of influenza.