AVIAN INFLUENZA SUSCEPTIBILITY IN *ALLIGATOR MISSISSIPPIENSIS*: A MODEL FOR INFLUENZA REPLICATION IN CROCODILIAN SPECIES

by

BRADLEY LAWRENCE TEMPLE

(Under the Direction of Travis Glenn)

ABSTRACT

Avian influenza has emerged as one the most ubiquitous viruses within our biosphere. It has been isolated from various species ranging from humans to mosquitoes. The emergence of H5N1and H1N1 has sparked worldwide interest in identifying and understanding which and how many species can be infected. Reptiles are an understudied class in regards to infectious diseases; however, recent research has begun to investigate the role these animals may contribute to viral ecology. Crocodilians have been around for approximately 240 million years and are regarded as the sister group to modern Aves. Therefore, crocodilians are a logical reptilian group to begin research with avian influenza isolates. A primary American alligator cell line along with alligator embryos were infected with four low-pathogenic avian influenza strains. This research has demonstrated the ability of wild-type avian influenza isolates to infect and replicate within a crocodilian system.

INDEX WORDS: Crocodilian, American alligator, Avian influenza, Reptile

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DEDICATION

I dedicate this to my wife and daughter. Without their help and support I might not have enjoyed myself quite as much as I have.

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CHAPTER 1

INTRODUCTION

The study of avian influenza virus (AIV) has increased dramatically during the past several years fueled by the emergence of the highly pathogenic strain H5N1. The H5N1 strain has been the cause of several poultry and human disease outbreaks in Asia. The first outbreak of H5N1 in humans occurred in Hong Kong in 1997 affecting 18 individuals, and claiming the lives of a 3-year-old boy, a 54-year-old man, and a 34-year-old woman (Mounts et al. 1999). Since then, H5N1 has infected at least 500 persons and claimed almost 300 lives (Organization 2010). In 2009, a novel H1N1 influenza strain with avian and swine components resulted in a pandemic that spread rapidly in humans across the world in a matter of weeks. H5N1 and the H1N1 pandemic has piqued interest in more broadly identifying susceptible species and possible vectors of transmission. The H1N1 pandemic of 2009 was the first pandemic declared by the World Health Organization (WHO) since SARS in 2002-2003 (Cherry 2004). Currently the H1N1 strain is still circulating, and the threat this disease poses is of great concern not only to humans but other animal species as well.

INFLUENZA A

Influenza A is a negative sense, single-stranded, enveloped RNA virus consisting of eight separate segments. It is classified within the *Orthomyxoviridae* family of viruses, which includes Influenza A, B, C, as well as, Thogotovirus, and Isavirus. The influenza genome consists of seven or eight genomic segments that encode for up to eleven viral proteins. Hemagglutinin (HA; encoded by segment 4), neuramindase (NA; encoded by segment 6), nucleoprotein (NP;

encoded by segment 5), M1 and M2 matrix proteins (encoded by segment 7), viral RNA polymerase (encoded by PB1, PB2, and PA encoded by segments 1,2, and 3, respectively) and the nuclear export proteins (NEP or NS2) and viral protein that may be involved in suppressing host interferon response (NS1; both encoded by segment 8) (Fields et al. 2007).

Additional information is given below concerning three of the above seven components (HA Matrix, and NP) utilized to assay for infection and replication within the model system used herein. The HA protein is the key protein involved in viral attachment to the cell membrane via binding to sialic acid residues on the periphery of the host membrane, and the eventual fusion with cellular endosomes that facilitate entry into the cell. HA present in avian influenza subtypes preferentially binds to sialic acid residues with α2,3 linkages; however, human influenza isolates preferentially bind to α2,6 sialic acid residues. The change in the HA sequence that is responsible for this preferential binding appears occur at amino acid position 226 (Fields et al. 2007). The importance of this is clearly evident when considering the ability of influenza to infect multiple species. Influenza segments have the ability to undergo re-assortment when two different quasi-species infect the same cell. This phenomenon was of great concern during the H1N1 outbreak of 2009-2010. It is believed that H1N1 arose from a re-assortment of pig and avian quasi-species that infected pigs and had genetic similarities to human influenza strains that allowed it to readily circulate rapidly within human populations causing illness and death (Smith et al. 2009).

Along with HA, NP is another integral component of influenza infection and replication within a host cell. NP has long been known to be one of the most abundant viral proteins present inside the cell during replication (Pons et al. 1969). NP is responsible for, amongst other things; encapsidating newly synthesized viral genomic RNA to be exported from the nucleus into the

cytoplasm that protects it from degradation by cellular RNAases. It has been proposed that NP plays a role in regulation of viral polymerase and the switch from transcription of viral mRNA to viral genomic RNA (Portela and Digard 2002).

Matrix proteins 1 and 2 are responsible for several components of viral infection and replication, including the completion of the un-coating process during infection, and the movement of new viral genome segments into the newly formed virons at the cell membrane during and after replication (Fields et al. 2007). M1 is ideal for assaying for viral replication because it is involved in the budding process of new virons from an infected cell. Additionally, this segment in the genome is highly conserved making it ideal for analysis of several different isolates.

The transmission of influenza between and amongst species is a highly researched area and while influenza A has been well documented in mammalian and avian species little is known about the presence of this virus in other taxa. Influenza is suspected of infecting insects, reptiles, and anurans, (Huchzermeyer 2002, Mancini et al. 2004, Barbazan et al. 2008, Davis and Spackman 2008), though the susceptibility of insects thus far is restricted to evidence to presently only confirmed in a mosquito cell line and from RT-PCR analysis from blood of engorged females (Barbazan et al. 2008). Influenza specific receptors have been identified in erythrocytes of two species of snakes and one species of frog that induced and inhibited hemagglutination of the virus (Mancini D.A.P. 2007). Crocodilians as well have been documented as having the potential to be susceptible to influenza A and C (Huchzermeyer 2002, Davis and Spackman 2008).

ALLIGATOR MISSISSIPPIENSIS

American alligators are poikilothermic reptiles in one of three families in the Order Crocodylia, a monophyletic assemblage (Huchzermeyer 2003). Alligators are just one of 23 extant species of Crocodilians (Gans 1969). The American alligator is dark grey to black in appearance with a broad flat snout. They inhabit fresh water habitats (swamps, lakes, lagoons and rivers) from eastern Texas through southern Florida and up into southeastern North Carolina (U.S. Fish and Wildlife Service. 1981)(Fig1). Although, alligators have been documented outside of their core geographic range described by the US Department of Interior, with individuals observed westward into lower parts of the Rio Grande and northward into Virginia (Ditmars 1910, Clarke 1953).

Alligators and crocodiles are two distinctly different monophyletic groups though their morphology and most physiology remain remarkably similar (Janke et al. 2005, Roos et al. 2007). Therefore it is important to look at diseases that have been documented in both alligators and crocodiles. Crocodilians are known to be susceptible to a variety of infectious diseases including West Nile Virus (WNV), Caiman pox, Crocodile pox, Adenoviral infection, Newcastle disease virus (Paramyxovirus), Eastern Equine Encephalitis virus, Influenza A and C, and Coronavirus (Huchzermeyer 2003, Klenk et al. 2004, Davis and Spackman 2008). All seven of these viruses documented in crocodilians have also been isolated in avian species either as active infections or as biological carriers (Ritchie 1995). Table 1.1 shows a list of the aforementioned viruses plus other viruses present in avian species that have not been investigated or documented in crocodilians. Along with these actively circulating viruses within both current human and animal populations, recent evidence has shown through genetic analyses the presence of endogenous inactive retroviruses in crocodilians (Jaratlerdsiri et al. 2009).

Previous research has established that avian species serve as natural reservoir for influenza A (Hubalek 2004, Krauss et al. 2007). However, it is important not only for other species of wildlife but humans as well that we identify potential reservoirs for this virus. American alligators are an understudied species that exist in both the wild and in captivity. They live in close proximity with various avian species and opportunistically feed on these animals, in addition to being exposed to their excrement. Alligators could routinely be exposed to avian influenza through feeding on infected birds, both through the ingestion of infected tissues and through nasal inhalation of infectious particles during the feeding process (Reperant et al. 2008). Along with opportunistic feeding in the wild, alligator farms feed their animals a diet that consists of fish, commercial alligator chow, beef and whole chickens (Peplow 1990). Alligators have been observed feeding on birds that fly into outdoor pens on the farms as well. Through real-time RT-PCR analysis, AIV has been identified in four crocodilian species *Alligator sinensis*, *Paleosuchus trigonatus*, *Caiman latirostris*, and *Crocodylus niloticus* (Davis and Spackman 2008). Filamentous forms of influenza C were identified using transmission electron microscopy in the feces of eight Nile crocodiles from a single farm in South Africa (Huchzermeyer 2003). In 2001 and 2002 over 1,250 alligators died on an alligator farm in southern Georgia of unknown causes (Miller et al. 2003). This unknown cause has since been hypothesized to be, at least for some of the cases, WNV. WNV may also be linked to the presence of subcutaneous lesions on the scales of American alligators. The lesions can render the hides unusable causing a dramatic effect on the economics of the alligator farming industry (Nevarez et al. 2008).

Alligators are also more closely related, in evolutionary terms, to avian species than any of the other non-avian species that influenza A has currently been isolated from (Wright et al.

1992, Barbazan et al. 2008, Reperant et al. 2009). However, current literature has possibly identified influenza A in several species of snakes (Mancini D.A.P. 2007). Therefore, it is reasonable to hypothesize that crocodilians, including but not limited to alligators, may be susceptible to avian viruses. Alligators and many avian species share several physiological features including egg structure and embryonic development (Deeming and Ferguson 1991). Likewise, studies have shown that the sequence of the insulin A chain is identical in alligators and chickens. Along with insulin, pancreatic polypeptides are similar in both species (Lance et al. 1984). In addition Reptilia and Aves along with Amphibia produce IgY antibodies, which are believed to be the precursor to IgG antibodies. IgY is still produced in many extant vertebrates including alligators and birds (Warr et al. 1995). These similarities in biological systems and the presence of environmental conditions conducive to influenza replication make alligators an important animal to investigate as a potential vector for AIV.

Our goal was to identify if crocodilians are susceptible to AIV. To accomplish this goal we used the American alligator (*Alligator mississippiensis*), as a model for AIV replication in crocodilian species and the role they may play as a reservoir or in the transmission of this disease. This study took a two-pronged approach at accomplishing this task by first investigating the ability of influenza A virus to infect and replicate in a primary alligator embryonic fibroblast cell line. We then examined the ability of influenza A virus to infect and replicate in both embryonated alligator eggs as well as in juvenile alligators.

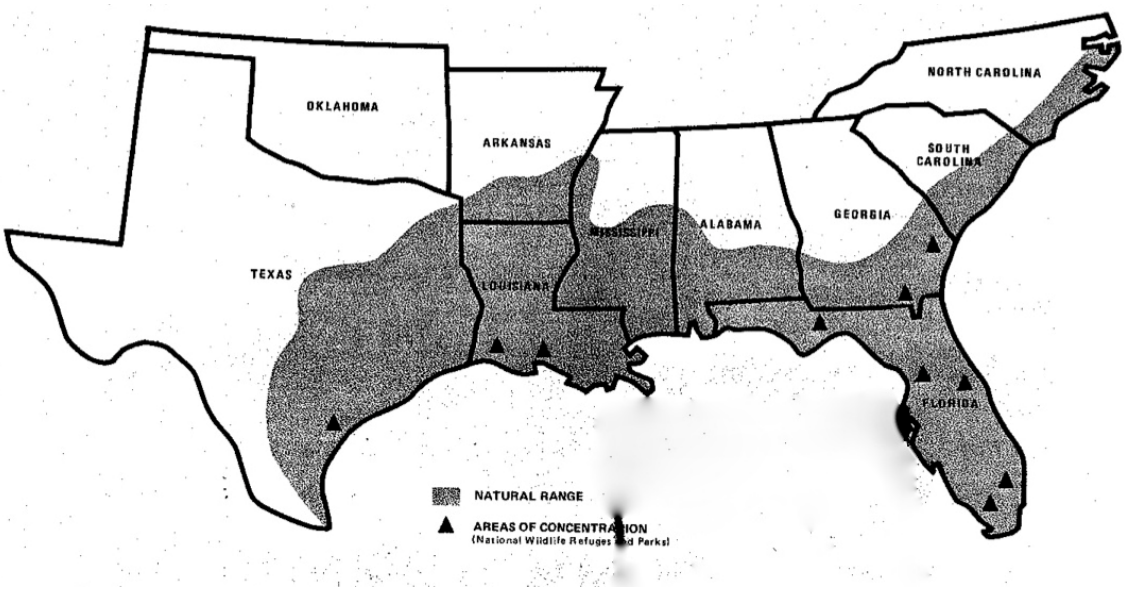

Figure 1.1: Geographic Distribution of *Alligator mississippiensis*. As described by Department of The Interior U.S. Fish and Wildlife document: "The American Alligator" August 1981.

Table1.1: Overview of Viruses Infecting Avian and Crocodilian Species. + indicates positive isolation of virus, - indicates no virus ,* indicates seroconversion but no clinical infection, Δ indicates virus isolated from excrement or from serum by PCR but no clear signs of infection documented. X indicates presence of endogenous retroviruses through PCR and sequence analysis but no evidence of viral activity has been documented. ? indicates unknown
1 see Ritche 1995
2,3 see (Huchzermeyer 2003, Davis and Spackman 2008, Nevarez et al. 2008, Jaratlerdsiri et al. 2009)

Virus	Avian[1]	Crocodilian[2,3]
Adenoviridae	+	+
Arenaviridae	*	?
Astroviridae	+	?
Birnaviridae	+	?
Bunyviridae	-	?
Circoviridae	+	?
Coronaviridae	+	Δ
Calciviridae	-	?
Filoviridae	-	?
Flaviviridae	+	+
Herpesviridae	+	?
Hepadnaviridae	+	?
Iridoviridae	-	?
Orthomyxoviridae	+	Δ
Papoviridae	+	?
Paramyxoviridae	+	*
Parvoviridae	+	?
Picornaviridae	+	?
Poxviridae	+	+
Reoviridae	+	?
Retroviridae	+	X
Rhabdoviridae	+	?
Togaviridae	+	*
Toroviridae	+	?

CHAPTER 2

MATERIALS AND METHODS

ANIMAL HUSBANDRY

Seven juvenile and three older alligators were housed in 0.36m^3 tanks. Water was supplied through a custom-built recirculation system that filtered out solid waste and food. Bio-balls and ammonia removal media were placed in the water recycling system to remove potentially harmful agents from the water. The aquatic temperature was kept at an average of 21°C. The juvenile gators were fed a mixture of commercial aquatic turtles pellet food, crickets and small goldfish purchased from local pet stores. The older (~2-3 year old) alligators were also fed a larger size commercial aquatic turtle pellet, in addition to whole chickens, chicken livers and gizzards, and large goldfish purchased at local pet supply stores. Each tank had a small underwater heater along with a UV light above the tank. All tanks had an accessible ledge above the water enabling the animals to get out of the water and bask under the UV light. All of these accommodations were in place to simulate, as much as possible, a natural habitat with the appropriate thermal gradient. The University of Georgia Institutional Animal Care and Use Committee approved all protocols and husbandry.

TISSUE CULTURE

A primary alligator embryonic fibroblast cell line was established by digesting a 41-51 day old embryo in a mixture of 1ml of Collagenase B (Roche Cat.#11-088-823-103) and 9 ml alpha-MEM cell culture media containing 1X antibiotics (10,000IU PenicillinG,10 μg/ml Streptomycin, 25 μg Amphotericin B/ml). The embryo was chilled overnight at 4°C then

dissected from the egg, and placed in a sterile petri dish washed with alpha-MEM and antibiotics. The embryo was dissected into ~ 1 mm x 1mm segments and washed three times in MEM antibiotic/antimycotic mixture. The tissue was then transferred to a 150 ml Erlenmeyer flask containing 10 ml of CollagenaseB mixed with an antibiotic/antimycotic (AB) solution with a magnetic stir bar. The tissues were digested at 80 revolutions per minute for three hours. The solution was then poured through a 70 μm cell strainer (BD Falcon Cat.#352350) washed with alpha-MEM/AB media and centrifuged at 1500x*g* for 15 minutes. A count of viable cells was obtained using trypan blue and a hemocytometer. Two T-75 tissue culture flasks were seeded with 500μl of MEM/AB, which contained 4.25 million cells, in 15 ml of cell culture media. After several attempts to find the most efficacious media formulation, alligator fibroblast cells were cultured in 175mM MEM (Gibco Cat.#11430), Primocin (100ug/ml Invivogen Cat.# antpm1), 1X L-glutamine (HyClone Cat.#SH-30034.01), 1X Penicillin/Streptomycin Amphotericin B (CellGro Cat.# 30-004-Cl), 10% FBS, 25mM Hepes (Gibco Cat.#15630-080), and 1X sodium bicarbonate (Gibco Cat.# 25080) pH 7.5 (Cuchens and Clem 1979). Cells reached confluency in five to seven days at 30°C under 6% CO_2 in a Thermo Electron Corporation (Waltham, Ma) Napco Series 8000DH CO_2 incubator.

EGG INOCULATION

Alligator eggs were collected from Rockefeller Wildlife Refuge (Cameron and Vermilion Parishes, LA, USA, 29°73°N, 92°82°W) and generously provided by Dr. Ruth Elsey of the Louisiana Department of Fish and Game. The eggs were housed in polystyrene chicken egg incubators filled with a mixture of moist vermiculite and peat moss at 28-30°C. De-ionized water was added to the incubators as needed to maintain ~90% humidity inside of the incubator.

Eggs were candled prior to inoculation with virus to determine viability. The primary determination of viability was visible movement of the embryo. Secondarily a high amount of vascularization visible inside the egg was determined to be sufficient evidence of embryo viability. A total of 52 alligator eggs and four LPAI strains, generously provided by Dr. David Stalknecht of the University of Georgia, were utilized in this study. Table 2.1 summarizes the following LPAI utilized in these experiments: A/chicken/Texas/167280-4/02 (H5N3), A/blue-winged teal/ Louisiana/1987 (H4N8), A/mallard/Minnesota/2008 (H2N3), and A/blue-winged teal/Louisiana/2007 (H3N8). The viruses were propagated in 9-11 day old embryonated chicken eggs. The stock titers were assayed in Madin Darby Canine Kidney cells (MDCK), and ranged from $10^{4.50}$ to $10^{6.24}$ $TCID_{50}$ /ml.

Eggs were segregated into four groups of 10 eggs in each infected group and one group of 12 control eggs. Each of the infected groups had five eggs incubated at 33°C and another five eggs at 36°C. The control eggs were divided into four sub-groups with three eggs in each group. Two of these groups were used as temperature controls and the other two were used as vehicle controls. The injection site was sterilized with 70% ethanol prior to inoculation with 200µl of 100-fold dilution of stock virus in PBS and antibiotics using a sterile 24 mm 18 gauge needle. Post-injection the site was sealed with Elmer's glue, and the eggs were placed in containers by group and incubated at given temperature. Eggs were incubated for a total of five days followed by overnight chilling at 4°C prior to embryo and allantoic fluid extraction. The eggs were removed from the 4°C and dissected using sterile techniques. The allantoic fluid from each egg was collected in 1.2 ml sterile cryovials. Three out of five embryos from each infected group and two out three from each control group were placed into 50 ml sterile conical tubes and filled with viral transport media (VTM) (MEM, 100X Antibiotic/Antimycotic, 50mg/ml Gentamicin,

50mg/ml Kanamycin, pH 7.4). The allantoic fluid samples and whole embryos were stored at -80°C. The two remaining embryos from each infected group and the remaining embryo from each control group had a small incision made from the cloaca to neck on the ventral side to expose the organs, and then placed into 50ml conical tubes filled with 10% formalin and fixed overnight. The formalin was removed the next day. The conical tubes were filled with fresh 10% formalin and the embryos were stored at room temperature until processed.

JUVENILE ALLIGATOR CHALLENGE STUDY

A total of seven juvenile alligators were used in a challenge study. Two alligators were used as uninfected controls and five juvenile alligators were challenged with LPAI H5N3 (A/chicken/Texas/167280-4/02) for a total of 72 hrs. Each animal was challenged with a total 3×10^4 infectious dose/ml. There were three routes of infection in each animal. Influenza A virus H5N3, diluted in PBS, was administered to the indicated sites in the following volumes: 10µl of a 1:2 dilution of 1×10^6 infectious dose/ml was injected into each nostril, 100µl of a 1:10 dilution of 1×10^6 infectious dose/ml was delivered orally down the trachea, and 100µl of 1:10 dilution of 1×10^6 infectious dose/ml was injected into the cloacae. The animals were housed in a bio-containment facility at the University of Georgia College of Veterinary Medicine. Each alligator was housed individually in large rat cages with micro-isolator lids. Each tank contained about 5cm of water, and had a platform placed inside to allow the animals to get out of the water. The ambient air temperature surrounding the tanks was 28-30°C. Sterile swabs of each nostril and occipital area, the cloaca, and an oral swab were taken every 24 hours. The swabs were placed in 1.2ml cryovials with 1ml of VTM. Likewise, 500µl water samples from the housing tanks were taken every 24hrs and placed into 500µl of VTM. All samples were stored at -80°C.

After 72 hrs, the animals were euthanized, according to proper animal use protocols, by being given an overdose of propofol (2ml at 10mg/ml) followed by cervical dislocation. The animals were dissected and all relevant organs (brain, trachea, lung, heart, liver, intestine, stomach, kidney, spleen, and pancreas) were removed and sectioned. One section was placed in 1.2ml cryovials with VTM and stored at -80°C. The other section was placed in 10% formalin to be fixed & embedded and stained for immunhistochemical analysis. The remainders of the animal carcasses were disposed of according to BSL2 protocols.

VIRAL TITRATION

Viral titration of allantoic fluid and tissues from embryonated alligator eggs; along with tissue and swab samples from the juvenile challenge study were assayed using a cell-based ELISA. All relevant tissues were extracted from both the juvenile gators and the embryos. The embryos were thawed at 4°C overnight prior to tissue collection. Each tissue was placed in a 2 ml collection tube with 1ml of VTM and lysed using a Qiagen Tissue Lyser at a setting of 30 oscillations per second for one minute. Each sample was centrifuged at 3,000x*g* for three minutes.

MDCK cells were plated out on 96-well micro-titer plates and incubated for 24 hrs at 37°C under 5%CO_2. Once plates had reached 80-90% confluency MDCK growth media was removed and the cells were washed three times with 1X sterile PBS. After washing, 180µl of viral infection media (MEM. 1X L-glutamine, 1X Penicillin/Streptomycin Amphotericin B, 500µl Gentamicin, and1µg/ml of TPCK trypsin) was added to each well. Twenty µl of sample was added in triplicate to wells in the top row followed by a serial 10-fold dilution in the remaining rows. The last row was left as a control row including both negative and positive controls. Positive controls were infected with a range of 500-750 $TCID_{50}$ units per well. The

plates were covered and incubated for either 36 or 72 hrs at 37°C under 5%CO_2. Following incubation, infection media was removed and the plates were washed three times with sterile 1X PBS prior to fixation in an 80:20 methanol/acetone mixture.

The plates were then blocked overnight at 4-8°C in 200µl of blocking solution per well (5% non-fat dry milk, 0.5% BSA, and 1X KPL wash buffer). Plates were washed three times with 300µl of 1X ELISA wash buffer (EWB) (1X PBS, Tween20, and Imidazole). One hundred µl of primary antibody (IgG Influenza A mouse anti-NP) was added to each well at a 1:500 dilution in blocking solution and incubated at room temperature for one hour. Plates were washed three times with EWB, and 100µl of a secondary AB (IgG HRP conjugate goat anti-mouse) was added to each well at a dilution of 1:10,000 in blocking solution. The plates were then wrapped in aluminum foil and rocked (low speed) and incubated at room temperature for one hour. The plates were then washed three times with EWB and 50µl of 1-Step Ultra TMB ELISA substrate solution (Pierce Cat.#34028) was added to each well. The reaction incubated for 15-20 minutes or until a blue color began to develop in the negative control wells. Sulfuric acid (50mL, 2M) was then added to stop the reaction. The plates were read at 450nm using a BioTek Powerwave plate reader, and analyzed using KC Junior (Bio-TEK Instruments Inc. Winooski, VT USA).

Tissue culture infectious dose at 50% ($TCID_{50}$) is a measure of the amount of infectious virus necessary to cause a pathological change in 50% of the culture. In this case 50% of the wells within each triplicate set at each viral dilution. $TCID_{50}$ was calculated by first measuring optical density (OD) in each well, and then subtracting out the background plus 2 standard deviations. Any wells that then had an OD reading greater than 0.05 were considered positive for virus. Using the Reed and Muench method a viral titer was established by calculating at which

titration greater than 50% of the wells were positive for infectious virus (Reed and Muench 1938). All statistical analysis was performed using Graph Pad Prism software and a Mann-Whitney test for statistical significance. The level of significance for all data was set at 0.05

HEMAGGLUTINATION ASSAY

The juvenile and embryo tissues along with a subset of allantoic fluid samples were assayed using hemagglutination assay. Supernatant (50μl) from each well of the infected MDCK plates was transferred to the corresponding well of a sterile 96 well round bottom plate. Then 50μl of 0.5% Turkey red blood cells was added to each well. The plates incubated for 30-40 minutes at room temperature before being observed for the presence/absence of hemagglutination.

IMMUNOFLUORESCENCE STAINING

Alligator fibroblast cells were cultured under the following conditions for all experiments unless otherwise stated. The cells were grown in T-150 tissue culture flasks at 30°C under 6%CO_2, and trypsinized using 0.05% Typsin_EDTA. Tissue culture treated plates (12-well) were seeded with 1.5×10^6 cells per well in 2ml alligator cell culture growth media. The plates were covered and incubated for 24 hrs. Once cells reached 80-90% confluency, the growth media was removed and 500μl of the appropriate viral dilution (1/100, 1/500, 1/1000) for each of the four virus strains was added to each well. The viruses were diluted in viral infection media (VIM), which consisted of normal alligator cell culture media without FBS and HEPES buffer and with the addition of exogenous TPCK-trypsin (1μg/ml). The plates were then incubated for three hours. The VIM was removed and the plates were washed twice with 1X sterile PBS, and 1ml of alligator cell growth media was added to each well. The plates were then incubated at 33°C under 6%CO_2 for 24 and 72 hrs and at 37°C under 6%CO_2 for 24 hrs. After incubation the cells

were washed once with 1X sterile PBS and fixed for 20 minutes with 80:20 methanol/acetone mixture. The plates were then allowed to air dry and stored at 4°C in 1X PBS until they were blocked overnight at 4°C in 1ml of blocking solution (5% FBS, 0.1% Tween20, and 1X KPL wash buffer). The blocking solution was removed and plates were washed three times with EWB. First, 500µl of primary antibody (IgG Influenza A anti-NP produced in mice) diluted 1:1500 in 1XPBS was added to each well and incubated for three hours on a plate rocker at room temperature. Plates were washed three times with EWB. Then, 500µl of secondary antibody (IgG goat anti-mouse FITC conjugated diluted 1:500 in 1XPBS was added to each well. Plates were wrapped in foil and incubated at room temperature on a plate rocker for one hour. The plates were washed three times with EWB and 500µl of 1µg/ml of 4',6-diamidino-2-phenylindole (DAPI) was added to each well. Plates were wrapped again in foil and incubated on plate rocker for 20 minutes at room temperature. Plates were washed once with EWB and the presence of viral antigen was determined by examination using a Zeiss Axiovert 40 CFL light microscope with the appropriate fluorescence microscopy filters.

TRANSMISSION ELECTRON MICROSCOPY

Alligator fibroblasts cells were plated on either six well non-treated tissue culture plates (Corning Cat.#3516) or fibronectin coated tissue culture plates (B.D Falcon Cat.#354402). A total of 10^6cell/well was added to each plate and incubated for 24hrs. After 24 hrs the cells were infected with a 500µl of 1×10^5/ml dilution of H5N3 in VIM. The plates were covered and incubated at 30°C under 6%CO_2. Cells were harvested at three time points post infection (30 minutes, six hours and 12 hrs.) After three hours the VIM was aspirated off and normal growth media was added to the cells. The cells were fixed at 4°C for one hour in 500µl of a modified Karnovsky's fixative (2% (para)formaldehyde,2% glutaraldehyde in 0.1M Cacodylate –HCL

buffer pH 7.23 (Karnovsky 1965). The cells were then scraped from the plates and transferred to a sterile 1.5ml centrifuge tube. The cells were centrifuged at 6,000x*g* for seven minutes. The fixative was removed and the pellet was re-suspended in 1ml of 0.1M Cacodylate buffer with 5% sucrose. The cells were centrifuged at 6,000x*g* for seven minutes and the wash buffer removed, and re-suspended again in one ml of 0.1M Cacodylate buffer with 5% sucrose (pH 7.23) and centrifuged at 6,000x*g* for seven minutes. The wash buffer was removed and the cells were re-suspended in 0.2M Cacodylate buffer pH 7.23 (Dawes 1971). The samples were the turned over to Mary Ard at the University of Georgia College of Veterinary Medicine Electron Microscopy laboratory for further preparation and analysis. Samples were placed in 1.5ml centrifuge tubes and enrobed in 3% noble agar at 58-60°C. They were then immediately centrifuged at 13,000x*g* in a Beckman microfuge for 10 minutes to condense the cells into a pellet. The samples were allowed to cool completely before removing the pellets (Dawes 1971). The agar-enrobed samples were placed in 0.1M Cacodylate-HCl buffer (pH 7.2).

Samples were then post fixed in 1% OsO_4 in 0.1M Cacodylate-HCl buffer (pH 7.2) for one hour at room temperature, washed four times for 10 minutes each time with de-ionized water, and dehydrated by transfer through a series of ethanol concentrations: 30%, 50%, 70%, 85%, 95%, and two changes in 100% for 15 minutes each. Samples were transferred to 100% acetone and 100% propylene oxide with two 10-minute and two 15-minute changes respectively. Samples were infiltrated in 1:1 propylene oxide-Epon 812 overnight at room temperature. The next day the samples were infiltrated twice for one hour each in 100% Epon 812 and then embedded in Epon 812 for 24 hrs at 60°C. Thin sections were cut with a diamond knife on an ultramicrotome and collected on 200-mesh copper grids. Grids were stained with 5% methanolic uranyl acetate and Reynold's lead citrate (Reynolds 1963) before examination. Grids were

examined on a JEM-1210 Transmission Electron Microscope (JEOL USA, Inc., Peabody MA) at an accelerating voltage of 120 KeV. Images were collected using the AMT XR41C Bottom-Mount CCD Camera. (Advanced Microscopy Techniques, Danvers, MA).

A one ml aliquot of H5N3 viral stock in chicken allantoic fluid was heat inactivated (95°C for 20 minutes) prior to preparation for negative stain EM analysis, which was performed by Mary Ard at the University of Georgia College of Veterinary Medicine Electron Microscopy Laboratory. A formvar-carbon coated 400-mesh grid was floated on a 40fl drop suspension of the sample. The grid was removed and excess sample was drained off using the edge of clean filter paper. The sample was floated on a drop of 3% aqueous phosphotungstic acid (PTA) (pH 7.0) for 30 seconds. A trace of Bacitracin was added to the PTA after pH as a wetting agent. After draining excess stain off grid, the grid was allowed to dry on filter paper (Hayat 1990). The sample was then viewed using a JEM-1210 Transmission Electron Microscope.

IMMUNOHISTOCHEMISTRY

Alligator tissues (brain, trachea, lung, heart, liver, intestine, stomach, kidney, spleen, and pancreas) previously extracted from both embryos and juveniles were fixed in 10% formaldehyde for 24 hrs. The formaldehyde was then removed and the tissues were stored in fresh 10% formaldehyde until staining. The tissues were sectioned and sent to the University of Georgia College of Veterinary Medicine Histology Laboratory to be embedded in paraffin and stained with haematoxylin and eosin for histology analysis. Unstained paraffin embedded tissues were stained using an IgG Influenza A anti-NP primary antibody at a 1:500 dilution, and a streptavidin-horseradish peroxidase labeled secondary. Lastly 3,3’-Diaminobenzidine (DAB) was added which reacted with the secondary antibody to produce a brown precipitate in antigen positive tissues.

REAL TIME RT-PCR

Total RNA was extracted from the liver and kidney from three out of the five embryos in each group, as well as from all of the juveniles using a Qiagen RNeasy Mini Kit (Qiagen Cat.#74106) according to the manufacturer's protocol. Along with these tissues a subset of allantoic fluid samples from infected and uninfected alligator eggs were extracted. The protocol used for RNA extraction in the tissues was modified slightly for all allantoic fluid samples. A 250µl aliquot of allantoic fluid was used in the extraction process rather than the standard amount set forth by the manufacture (Spackman 2008). Total RNA (50µl) was extracted from each sample and quantified on a BioPhotometer. The total RNA concentrations ranged from 4-402µg/ml.

cDNA synthesis and subsequent real-time RT-PCR were performed using a Qiagen One Step RT-PCR Kit (Qiagen Cat.#210212) on a Stratagene MX3000p or 3005p thermocycler. The primers used were specific for M gene genomic RNA sequence M+25 forward (AGA TGA TTC TAA CCG AGG TCG) M-124 reverse (TGC AAA AAC ATC TTC AAG TCT CTG) including the internal probe (M+64 [FAM]-TCA GGC CCC CTC AAA GCC GA-[BHQ-1]) (Spackman 2008). The total volume per reaction was 25µl reaction with three µl of template RNA per reaction. Each sample was run in triplicate with WSN influenza virus used as positive control. Molecular grade water along with uninfected tissue and allantoic fluid were used as negative controls. The thermocycling conditions for the reverse transcription step were setup per manufactures instructions: one cycle for 30 minutes at 50°C, one cycle for 15 minutes at 95°C The PCR conditions were: Denaturation 30 seconds at 94°C, Annealing one minute at 60°C, and Extension one minute at 72°C for 45 cycles. The reaction was then held at 72°C for an additional 10 minutes. The results were analyzed using Stratagene Q-PCR software.

ALLIGATOR IMMUNE RESPONSE

Blood was drawn from either the caudal vein or the common carotid artery (Reese 1914) with an 18ga ¾ inch needle from a 2-3 year old alligator prior to injection with purified inactivated influenza A/Puerto Rico/8/34 (PR8) from Charles River. A 400µl injection (200µl incomplete Freunds adjuvant, 100µl sterile PBS, 100µl PR8 2mg/ml) was given intraperitoneal (I.P.) with a sterile 25gauge needle. A second injection was given 51 days at the same concentration in the same volume. Blood was collected 35 days post-first injection and 17 days post-second injection. An aliquot of whole blood (500µl) was transferred to a capillary blood sampling tube (Capiject T-MG) and incubated at room temperature for 20-30 minutes. After clot formation the samples were centrifuged for seven minutes at 5,000x*g.* The serum was transferred to a 1.5ml tube and stored at -20°C until use.

Two 96-well flat bottom high binding H2B (Falcon) plates were coated with a 1/100 dilution of either A/PR/8/34 or A/X-31 influenza strain in PBS and incubated for 24 hrs at 4°C. The plates were removed from 4°C and blocked for one hour in 200µl of blocking solution (5% FBS, 0.1% Tween20, and ELISA wash buffer) at room temperature. The plates were then washed three times with EWB prior to the addition of 1:50 dilution of serum of each sample (pre-immune, first vaccination, and second vaccination) in blocking solution (125µl) to the top row of each plate followed by two-fold serial dilutions in the remaining rows. The plates were incubated for one hour at room temperature and then washed with three times with EWB. One hundred µl of a 1:1000 dilution of Goat Anti-alligator IgG(IgY) antibody(US Biological Cat.#A1358-05C) in blocking solution was added to each well. The plates were incubated at room temperature for one hour and then washed three times with EWB. Following washes 100µl of 1:1000 dilution of phosphatase labeled Rabbit Anti-goat IgG (KPL Cat.#15-13-06) in

blocking solution was added to each well and incubated for one hour at room temperature. Plates were then washed three times with EWB prior to incubation for two hours in 100µl of p-Nitrophenylphosphate (KPL Cat.#50-80-00). The plates were read at 415nm using a BioTek Powerwave plate reader, and analyzed using KC Junior (Bio-TEK Instruments Inc. Winooski, VT USA).

Table2.1: Low Pathogenicity Avian influenza Virus Strains. Viral strains used to infect alligator eggs and alligator cell lines. Blue winged teal (BWTE) Mallard Duck (MALL), Chicken (CHK).

Formal Name	ID	Sub-type	Species	Location	Year Collected
A/blue-wingedteal/Louisana/1987	3B	H4N8	BWTE	Cameron Parish, LA	1987
A/mallard/Minnesota/2008	A108-3849	H2N3	MALL	Marshall County, MN	2008
A/blue-winged teal/Louisana/1987	A107-5839	H3N8	BWTE	Cameron Parish, LA	2007
A/chicken/Texas/167280-4/02	A167280	H5N3	CHK	Texas	2002

CHAPTER 3

RESULTS

Previous evidence has indicated the presence of genomic elements of AIV in alligator serum and the presence of influenza C in crocodilian feces (Huchzermeyer 2003, Davis and Spackman 2008). The goal of this study was to empirically determine if AIV could infect and replicate within a crocodilian system. We approached this question by first infecting primary embryonic alligator fibroblast cell line with each of the four LPAI. Secondly, we challenged both embryonated alligator eggs and juvenile alligators with LPAI.

IN-VITRO INFECTION OF PRIMARY EMBRYONIC FIBROBLASTS

To determine if alligator cells could be infected with AIV primary embryonic fibroblasts were generated and infected with one of four LPAI strains. After infection primary embryonic alligator fibroblasts were positive for the presence of viral nucleoprotein in each of the four viral strains at multiplicity of infections (MOI's) ranging from 0.0006-0.1PFU/cell (Figures 3.1-3.4). The NP antigen was present within the cytoplasm but was more strongly localized within the nucleus. The nucleus was identified using DAPI, a fluorescent molecule that binds dsDNA. Cells infected with each of the four viral strains were positive for NP antigen at 33°C and 37°C 24 hrs post-infection. Likewise, all four viral strains were positive for viral antigen at 33°C 72 hrs post-infection, though it was present in varying degrees amongst the four strains. The staining was dim in H2N3 or H4N8. In contrast NP antigen was more evident with H5N3 or H3N8 strains 72 hrs post-infection. In summary immunostaining with NP antibody demonstrated that alligator fibroblasts were susceptible to infection with all four LPAI strains at temperatures consistent for

both embryo growth and development and virus replication. In order to further demonstrate infection we utilized electron microscopy to visualize the virus entering and/or exiting the cell.

ULTRA-STRUCTURE OF PRIMARY EMBRYONIC FIBROBLASTS

Electron microscopy was used in an attempt to observe the virus interaction inside of the cell. Figure 3.5 shows a negative stain image of the H5N3 virus used to infect the alligator cell line, juvenile alligators, and the embryonated alligator eggs. Figure 3.6 shows the ultra-structure of an embryonic alligator fibroblast cell from culture that was infected with the LPAI H5N3 virus at an MOI of 0.5PFU/cell. The cells were fixed 30 minutes post-infection. The arrows indicate possible virus entering into the cell via the endocytosis near the plasma membrane.

INOCULATION OF EMBRYONATED ALLIGATOR EGGS

After observing infection in an alligator cell line, the next step was to investigate whether viral replication occurs in an *in ovo* system. This experiment was accomplished by first inoculating embryonated alligator eggs with each of the four strains of LPAI followed by measuring viral titers in the allantoic fluid five days post-infection. Eggs were incubated at two temperatures 33°C and 36°C. The significance of these two temperatures being that 33°C is the highest optimal temperature for alligator embryo development in the wild and 36°C provided the closest optimal temperature for viral replication that could be used without being fatal to the alligator embryos (Smith 1975). The viral titers were measured using a cell-based ELISA assay with a level of detection of $10^{2.00}$ $TCID_{50}$/ml. All control groups (temperature and vehicle) performed as expected and showed no detectable level of virus.

Viral activity exceeded input levels in all four strains indicated by viral titer levels in allantoic fluid ranging from $10^{2.00}$ to $10^{7.75}$ $TCID_{50}$/ml (Figure3.7). The H5N3, H3N8, and H4N8 strains, while showing clear evidence of replication, did not show statistically different

amounts of virus ($p<0.05$) when comparing viral replication at 33°C vs. 36°C. The H2N3 strain was the only strain that showed a significant difference in viral replication when comparing the two temperatures. The data suggests that this strain replicates significantly better in embryonated alligator eggs at 36°C vs. 33°C.

The H5N3 ($p> 0.05$), H4N8 ($p>0.05$), and H3N8 ($p>0.05$) strains of influenza virus incubated at 33°C had mean viral titers of $10^{4.79}$ (±0.77), $10^{3.84}$ (±0.50), $10^{3.59}$ (±0.55) $TCID_{50}$/ml respectively, while at 36°C these three strains had mean viral titers of $10^{4.84}$ (±0.65), $10^{4.35}$ (±0.15), $10^{4.39}$ (±0.57) $TCID_{50}$/ml respectively. These data indicate that temperature had no significant effect on viral replication of these viral strains when cultured in alligator eggs.

While the previous three strains did not demonstrate a significant change in replication at a higher temperature, the H2N3 strain proved to be the most temperature sensitive with respect to viral replication when grown in embryonated alligator eggs. The 33°C group had a mean viral titer of $10^{3.49}$ (±0.67) $TCID_{50}$/ml vs. $10^{6.04}$ (±0.67) $TCID_{50}$/ml in the 36°C group resulting in a significant increase in viral replication ($p<0.05$) when compared to the 33°C group. In order to validate these results, a subset of allantoic fluid samples were assayed in a standard HA assay. The virus titer, as measured by HA assay, corresponded almost directly with the levels measured by cell-based ELISA (Data not shown).

The presence of new viral progeny was indicated by the production of NP protein and HA activity. In addition viral replication was confirmed by examining production of viral genomic RNA. This task was accomplished by assaying a subset of allantoic fluid samples (n=5) from the 36°C alligator eggs using real-time RT-PCR with primers specific to M gene genomic RNA.

Figure 3.8 shows the mean C_t values from allantoic fluid samples. All four viral strains were below the threshold level indicated by the mean C_t value of 34.79 (±0.17) derived from the allantoic fluid of the uninfected alligator eggs. In general the level of M gene genomic RNA in each sample corresponded to the data presented in the viral titration samples. H5N3 samples assayed had a mean C_t value of 13.97 (±0.15). Both the H3N8 and the H4N8 samples had mean C_t values of 15.38 (±0.13) and 15.25 (±0.06) respectively. The H2N3 samples had the lowest mean C_t value 13.23 (±0.27). A/WSN/33 (H1N1) was used as a positive control [15.67 (±0.37)]. These results confirmed the presence of both infectious virus (NP and HA) and viral genomic RNA (M gene) in the allantoic fluid further supporting the hypothesis that the AIVs were replicating in infected alligator eggs.

IMMUNOHISTOCHEMISTRY OF EMBRYONIC TISSUES

After confirmation of infection and virus replication of all four AIV strains in embryonated alligator eggs at 33°C and 36°C it had to be determined in which tissues viral replication was occurring. All organs were extracted in two out of five embryos from each group for both H&E and immunohistochemical detection. Upon examination all mock-infected embryos were negative for lesions, congestion, and necrosis in all tissues and had no indications of antigen staining. However, one of the vehicle control 36°C embryos had evidence of fluid in the lungs that resulted in non-specific staining, and was excluded from analysis. In contrast, the alligator eggs infected with each of the four viral strains had NP staining predominantly in liver and kidneys and in accordance with previous results had a higher level of staining at 36°C (Table 3.1).

The H4N8 inoculated embryos incubated at 33°C had congestion in liver and kidney in one embryo and the second embryo had congestion in the liver only. Both embryos had staining

present in the liver and the kidney as well as some staining in the lungs (n=1). Embryos incubated at 36°C unlike the latter group had wide spread necrosis (Figure 3.9). However, one embryo had bacteria present in the cervical region, which may have contributed in part to the necrosis. These embryos as seen in the 33°C group, were positive for NP antigen in both liver and kidneys (Figures 3.9-3.10) and had staining present in the lungs of one embryo.

The H3N8 inoculated embryos incubated at 33°C had no lesions, congestion, or necrosis present in any tissues. There was weak viral antigen staining in the lung and kidney of one, and in the liver of the second. Macrophages were also present in the embryo in which the lung and kidney were positive for viral antigen, indicative of an innate immune response. The 36°C embryos, unlike the 33°C embryos had congestion in the liver of one embryo and a focal area of liver necrosis in the other. Both embryos were positive for NP antigen in the liver and kidneys.

The H5N3 inoculated embryos incubated at 33°C presented with no lesions, congestion or necrosis, but there was NP antigen detected in the liver and intestine of one embryo. The second embryo had NP detected in the liver only. One of the 36°C embryos had wide spread bacterial contamination and necrosis. The bacterial contamination present hindered our ability to assess what effect the virus had on the animal. The other embryo had histological results similar to the 33°C group. The animals presented with no lesions, congestion, or necrosis in any tissues. Unlike in the lower temperature group NP was detected in both liver and kidney.

The H2N3 inoculated embryos unlike in previous assays did not present with the strongest amount of evidence for virus replication. They presented with no necrosis or lesions upon examination in either group. The 33°C group (n=1) presented with congestion in the liver

and the other embryo had weak staining in the liver. Both embryos in the 36°C group were positive for staining in the kidney vs. no staining of the kidney in the 33°C group

The IHC results clearly indicated the presence of NP protein in embryonic tissues. There was some indication of diseased tissue evident from the presence of lesions, necrosis, and congestion in the liver and kidneys. Based on these results the primary sites of infection were determined to be the liver and kidney.

M GENE PRESENCE IN EMBRYONIC TISSUES

The results presented in Table3.1 indicated that liver and kidney were the predominant sites of virus replication. Nonetheless after assaying by both cell-based ELISA ($TCID_{50}$) and HA there was no detectable level of infectious virus or viral protein in either the control or infected alligator tissue. Due to the conflicting results obtained from the IHC and the $TCID_{50}$ and HA assays we turned to a more sensitive technique of virus detection. Total RNA was extracted from kidney and liver from the three remaining embryos in each group along with the remaining control embryos that had not been employed for the IHC assay.

The mean control C_t values from the uninfected embryonic tissues were used to calculate the negative threshold value (36.40). The control values for the embryos incubated at 33°C were 40.00 (±1.03) for kidney (n=9) and 42.19 (± 1.14) for liver (n=9). Embryos incubated at 36°C had similar values of 40.65 (±1.03) for kidney (n=12) and 41.80 (± 0.75) for liver (n=12). As indicated in figures 3.11-3.14 the mean C_t value of either or both liver and kidney samples from embryos infected with one of four viral strains at 33°C or 36°C was below the negative threshold indicating the presence of M gene genomic RNA. In three out of four viral strains (H4N8, H3N8, and H5N3), there did not appear to be a substantial difference between the embryos inoculated at 33°C vs. 36°C (Figures 3.11-3.13). In contrast, the H2N3 strain in

followed a similar trend seen in the allantoic fluid samples where there was an increase in viral M gene genomic RNA at 36°C vs. 33°C as indicated in Figure 3.14.

The results of the RT-PCR analysis from H4N8 infected groups are presented in Figure 3.11. The 33°C infected group had a mean C_t value 34.92 (± 0.69) for kidney (n=9) and 34.78 (±0.44) for liver (n=9) vs. a mean C_t value 34.45 (± 0.67) for kidney (n=9) and 33.68 (±0.69) for liver (n=9) in the 36°C group. These results indicate that viral genomic RNA was present in both tissues at both temperatures in fairly equal concentrations. This suggests that temperature did not play a significant role in the production of M gene genomic RNA.

The H3N8 33°C infected group had a mean C_t value 36.24 (± 0.66) for kidney (n=9) and 36.87 (±0.38) for liver (n=9) vs. 34.27 (± 1.35) for kidney (n=9) and 33.58 (±0.59) for liver (n=9) in the 36°C group as presented in Figure 3.12. The results from the H3N8 group indicate that temperature played a small role in genomic RNA production because both the kidney and liver tissues at 33°C had mean C_t values equal to the negative threshold. However, both kidney and liver had mean C_t values below the negative threshold.

Figure 3.13 illustrates the results collected from RT-PCR analysis of liver and kidney for the H5N3 infected eggs. The 33°C infected group had a mean C_t value 36.21 (± 0.37) for kidney (n=9) and 34.38 (±1.14) for liver (n=9) vs. 33.32 (± 1.12) for kidney (n=9) and 36.48 (±0.49) for liver (n=9) in the infected group 36°C. These observations follow similar trends as seen in the viral titer results from the allantoic fluid in which temperature did not play a significant role in viral replication. The mean C_t values in the kidney was at the threshold for 33°C, but below threshold at 36°, where as, the liver had a mean C_t value below threshold at 33°C and right at the threshold at 36°C. These results demonstrate that the level viral

replication was not increase or decreased when comparing these two temperatures in embryonated alligator eggs.

The H2N3 results are presented in Figure 3.14. The 33°C infected group had a mean C_t value 34.40 (± 0.70) for kidney (n=9) and 31.88 (±0.39) for liver (n=9) vs. a mean C_t value 30.63 (± 1.04) for kidney (n=9) and 29.73 (±0.46) for liver (n=9) in the infected 36°C group. All four mean C_t values were below the threshold indicating the presence of viral genomic RNA in both liver and kidney at both temperatures. While both tissues indicated the presence of viral genomic RNA there appears to be higher levels of viral RNA in both tissues at 36°C vs. 33°C. The H2N3 real-time RT-PCR results support the data collected from the cell based ELISA that the H2N3 strain replicated more efficiently at 36°C vs. 33°C.

Since the levels of progeny virus were below the limit of detection for $TCID_{50}$ and HA assay, the real-time RT-PCR assay was employed to confirm the presence of virus and viral replication in the tissues suggested through IHC analysis. Although, the levels of infectious virus in the tissues were low, the presence of genomic RNA (identified with M gene specific primers) and NP protein (confirmed with IHC) confirms that alligator embryos are susceptible to infection and can support aspects of viral replication.

VIRUS REPLICATION IN JUVENILES CHALLENGED WITH LPAI H5N3

The next step in determining the susceptibility of the American alligator to avian influenza viruses was to challenge juvenile alligators to determine if they were different in their response, compared to embryos, when exposed to AIV. The H5N3 strain was selected because previous results indicated that its replication was less temperature dependent than the other three strains. Juvenile alligators (1-1.5 years old) were inoculated through three routes of infection (Intranasal, Intracloacal, and Oral pharyngeal) with 3×10^4 PFU/ml with

A/chicken/Texas/167280-4/02. No virus or viral antigen was detected in any of the juvenile tissue or swab samples by $TCID_{50}$ (cell-based ELISA), immunohistochemistry, or hemagglutination assay. Additionally there was no detectable level of viral genomic RNA in any of the liver or kidney samples assayed by real-time RT-PCR. The results from the RT-PCR as identified in Figure 3.15 for the juvenile alligators had a mean C_t value of 40.32 (±1.64) for the uninfected kidney samples (n=2) vs. 41.53 (±0.68) for infected kidney samples (n=5). The liver samples produced similar values with the uninfected samples (n=2) producing a mean C_t value of 42.92 (±1.13 vs. 39.61 (±0.49) for infected samples (n=5). All these values were above the negative threshold (36.40). The assay worked as expected using WSN influenza strain as a positive control [mean C_t value of 16.95 (±0.30)]. Along with tissues and swab samples all water samples were negative for both NP protein and hemagglutination activity.

HUMORAL IMMUNE RESPONSE IN AMERICAN ALLIGATORS

The knowledge surrounding immune responses in alligators is limited (Gans 1969, Warr et al. 1995, Origgi 2002). An injection with inactivated PR8 produced a humoral immune response in the alligator, as indicated by the production of influenza A specific antibodies. The data presented in Figure 3.16 and 3.17 show that the alligator produced an antibody response to both X31 (H3N1) and PR8 (H1N1) above background 1:800 (PR8) and 1:400 (X31). An antibody response was observed at serum dilutions of 1:800 in the first vaccination and 1:1600 in the second vaccination for the X31 strain. The PR8 indicated an immune response at serum dilutions of 1:3200 after the first vaccination and 1:6400 after the second vaccination. While the background levels for the pre-immune sera were high. There was a clear increase in immune response above background, suggesting alligators can produce cross-reactive anti-NP antibodies against influenza A and a second exposure to antigen will boost the antibody titer.

The sum of these results clearly indicates that American alligators may be susceptible to infection with avian influenza viruses. The information presented indicates that AIV can infect alligator fibroblast-like cells, embryos and embryonated eggs (i.e. chorioallantoic memebrane). The virus clearly replicates within the eggs to high titers. While we did not see active replication in the juvenile alligators, this could be due to the limitations of the animal husbandry conditions (e.g. temperature restrictions) or other unidentified variables. Nevertheless, these strains, which were isolated from avian species in areas overlapping alligator home ranges can replicate to significantly high levels within a crocodilian system even at temperatures less than optimal for viral replication.

Table 3.1: **Immunohistochemistry from embryonic tissues**. Summary of IHC data from embryos extracted from inoculated alligator eggs. Control embryos n=4. Infected embryos n=4 per viral strain. L=Liver, Lu=Lung, K=Kidney. *indicates one control embryo in the vehicle control 36°C group that inhaled fluid which resulted in non-specific staining(False positive) Bacteria indicates bacterial contamination present in tissues.

Viral Strain	Head	Neck	Chest	Abdomen	NP Antigen
Control*	-	-	-	-	-
H3N8 33°C	-	-	-	-	Lu,L,K (1/2)
H3N8 36°C	-	-	L.congestion(1/2) L.necrosis (1/2)	-	L,K (2/2)
H4N8 33°C	-	-	L. congestion (2/2)	K.congestion (1/2)	Lu (1/2) L,K(2/2)
H4N8 36°C	Necrosis(2/2)	Necrosis(2/2) Bacteria(1/2)	Necrosis (2/2)	Necrosis(2/2)	Lu (1/2) L,K (2/2)
H5N3 33°C	-	-	-	-	L,K,I (1/2)
H5N3 36°C	Necrosis(1/2) Bacteria(1/2)	Necrosis(1/2)	Necrosis(1/2)	Necrosis(1/2)	L,K (1/2)
H2N3 33°C	-	-	L. congestion (1/2)	-	L (1/2)
H2N3 36°C	-	-	-	-	L,K (2/2)

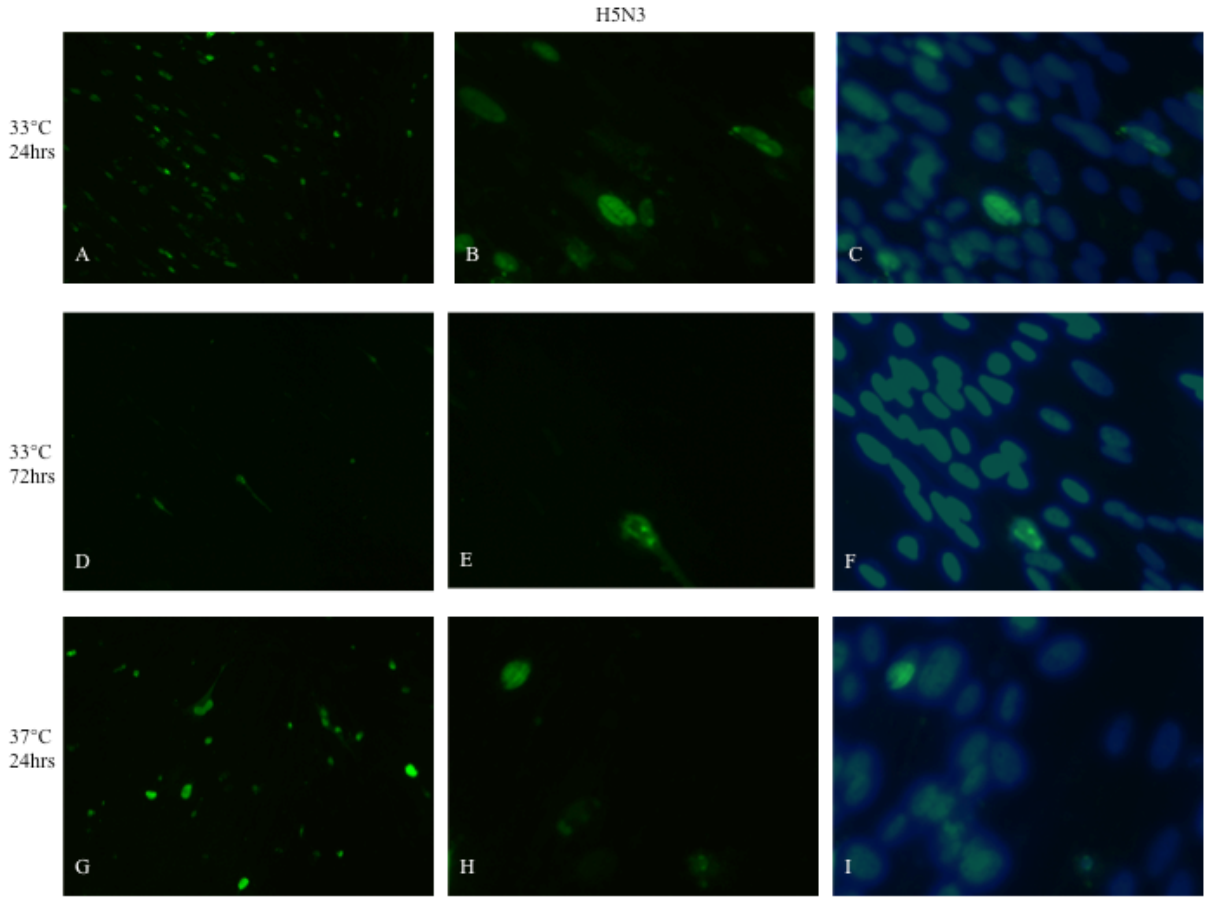

Figure 3.1: Immunofluorescent staining of NP in primary alligator fibroblast infected with H5N3. The immunostaining was done using a FITC conjugated goat anti-mouse NP antibody of alligator fibroblast cells infected with A/Chicken/Texas/167280-4/02 (H5N3). Green fluorescent indicates presence of viral nucleoprotein. The presence of dsDNA in the nucleus is indicated by a blue fluorescent image obtained by staining dsDNA in the nucleus with 4',6-diamidino-2-phenylindole (DAPI). (A): indicates positive staining of NP antigen at an MOI of 0.02 24 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (B): indicates positive staining at an MOI of 0.02 of NP antigen 24 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (C): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (D): indicates positive staining of NP antigen at an MOI of 0.01 72 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (E): indicates positive staining of NP antigen at an MOI of 0.01 72 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (F): indicates co-localization of dsDNA and NP antigen 72 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (G): indicates positive staining of NP antigen at an MOI of 0.01 24 hours post-infection at 37°C under 6% CO_2 at 10X magnification. (H): indicates positive staining of NP antigen at an MOI of 0.01 24 hours post-infection at 37°C under 6% CO_2 at 40X magnification. (I): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 37°C under 6% CO_2 in the nucleus under 40X magnification.

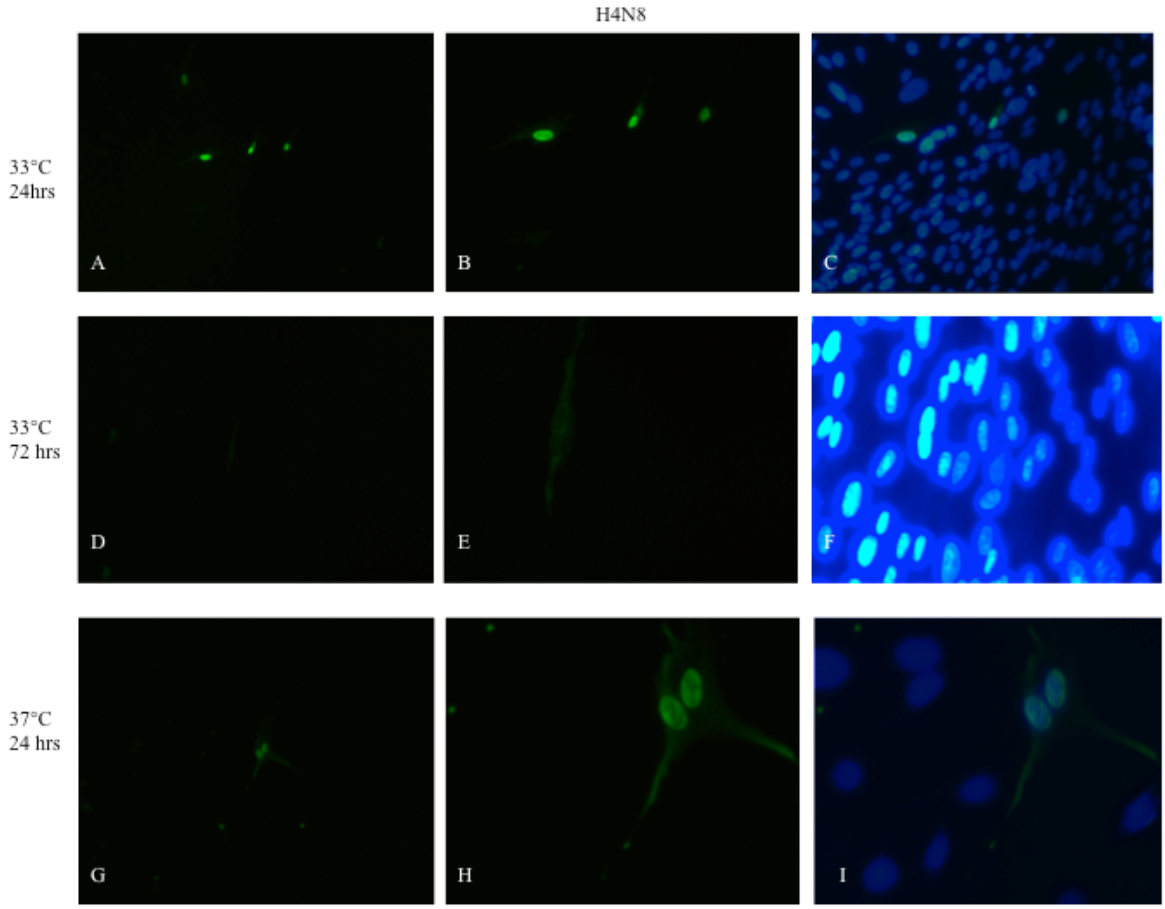

Figure 3.2: Immunofluorescent staining of NP in primary alligator fibroblast infected with H4N8. The immunostaining was done using a FITC conjugated goat anti-mouse NP antibody of alligator fibroblast cells infected with A/blue-winged teal/ Louisana/1987 (H4N8). Green fluorescent indicates presence of viral nucleoprotein. The presence of dsDNA in the nucleus is indicated by a blue fluorescent image obtained by staining dsDNA in the nucleus with 4',6-diamidino-2-phenylindole (DAPI). (A): indicates positive staining of NP antigen at an MOI of 0.01 24 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (B): indicates positive staining of NP antigen at an MOI of 0.01 24 hours post-infection at 33°C under 6% CO_2 at 20X magnification. (C): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 33°C under 6% CO_2 in the nucleus under 20X magnification. (D): indicates very faint positive staining of NP antigen at an MOI of 0.005 72 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (E): indicates very faint positive staining a of NP antigen at an MOI of 0.005 72 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (F): indicates presence of dsDNA 72 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (G): indicates positive staining of NP antigen at an MOI of 0.01 24 hours post-infection at 37°C under 6% CO_2 at 10X magnification. (H): indicates positive staining at an MOI of 0.01 of NP antigen 24 hours post-infection at 37°C under 6% CO_2 at 40X magnification. (I): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 37°C under 6% CO_2 in the nucleus under 40X magnification.

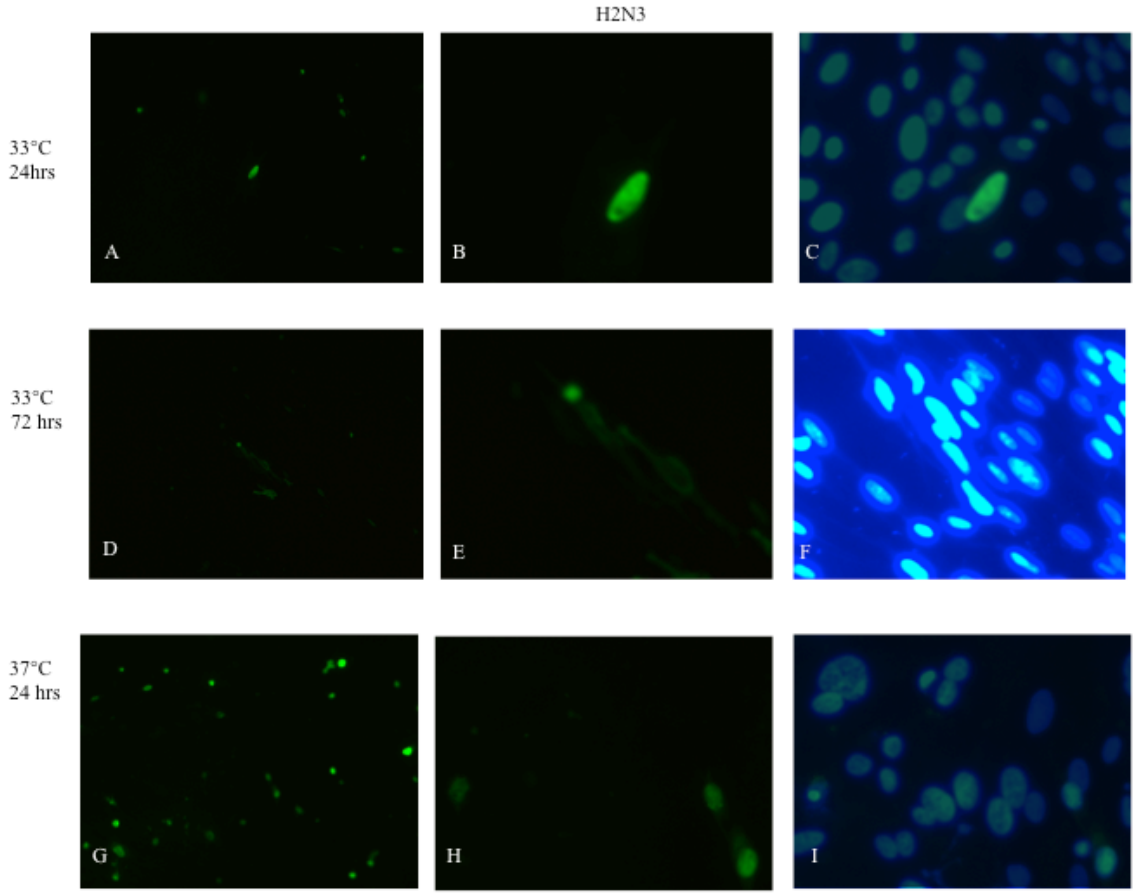

Figure 3.3: Immunofluorescent staining of NP in primary alligator fibroblast infected with H2N3. Immuonostaining was done using a FITC conjugated goat anti-mouse NP antibody of alligator fibroblast cells infected with A/mallard/Minnesota/2008 (H2N3). Green fluorescent indicates presence of viral nucleoprotein. The presence of dsDNA in the nucleus is indicated by a blue fluorescent image obtained by staining dsDNA in the nucleus with 4',6-diamidino-2-phenylindole (DAPI). (A): indicates positive staining of NP antigen at an MOI of 0.0006 24 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (B): indicates positive staining of NP antigen at an MOI of 0.0006 24 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (C): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (D): indicates positive staining of NP antigen at an MOI of 0.003 72 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (E): indicates positive staining of NP antigen at an MOI of 0.003 72 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (F): indicates presence of dsDNA 72 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (G): indicates positive staining of NP antigen at an MOI of 0.0003 24 hours post-infection at 37°C under 6% CO_2 at 10X magnification. (H): indicates positive staining of NP antigen at an MOI of 0.0003 24 hours post-infection at 37°C under 6% CO_2 at 40X magnification. (I): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 37°C under 6% CO_2 in the nucleus under 40X magnification.

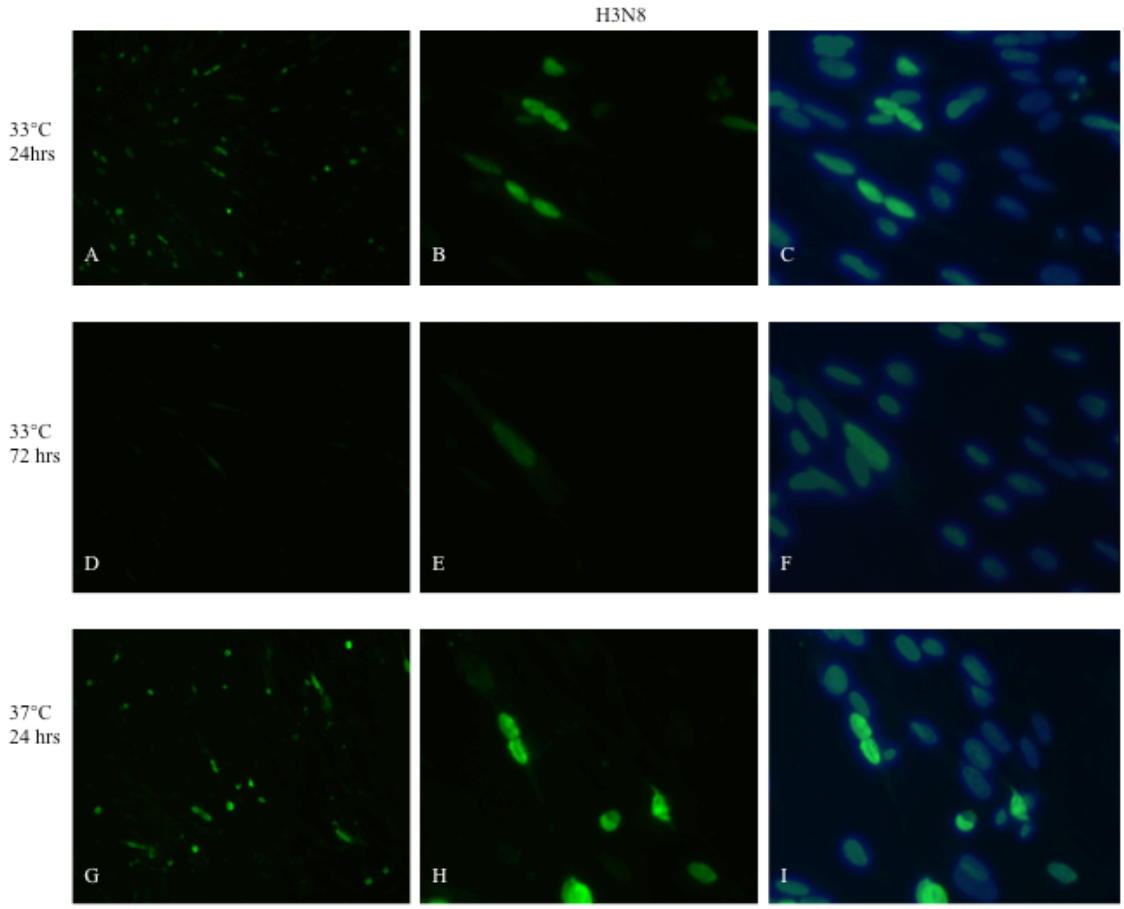

Figure 3.4: Immunofluorescent staining of NP in primary alligator fibroblast infected with H3N8. Immunostaining was done using a FITC conjugated goat anti-mouse NP antibody of alligator fibroblast cells infected with A/blue-winged teal/Louisana/2007 (H3N8). Green fluorescent indicates presence of viral nucleoprotein. The presence of dsDNA in the nucleus is indicated by a blue fluorescent image obtained by staining dsDNA in the nucleus with 4',6-diamidino-2-phenylindole (DAPI). (A): indicates positive staining of NP antigen at an MOI of 0.05 24 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (B): indicates positive staining of NP antigen at an MOI of 0.05 24 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (C): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (D): indicates positive staining of NP antigen at an MOI of 0.005 72 hours post-infection at 33°C under 6% CO_2 at 10X magnification. (E): indicates positive staining of NP antigen at an MOI of 0.005 72 hours post-infection at 33°C under 6% CO_2 at 40X magnification. (F): indicates co-localization of dsDNA and NP antigen 72 hours post-infection at 33°C under 6% CO_2 in the nucleus under 40X magnification. (G): indicates positive staining of NP antigen at an MOI of 0.05 24 hours post-infection at 37°C under 6% CO_2 at 10X magnification. (H): indicates positive staining of NP antigen at an MOI of 0.05 24 hours post-infection at 37°C under 6% CO_2 at 40X magnification. (I): indicates co-localization of dsDNA and NP antigen 24 hours post-infection at 37°C under 6% CO_2 in the nucleus under 40X magnification.

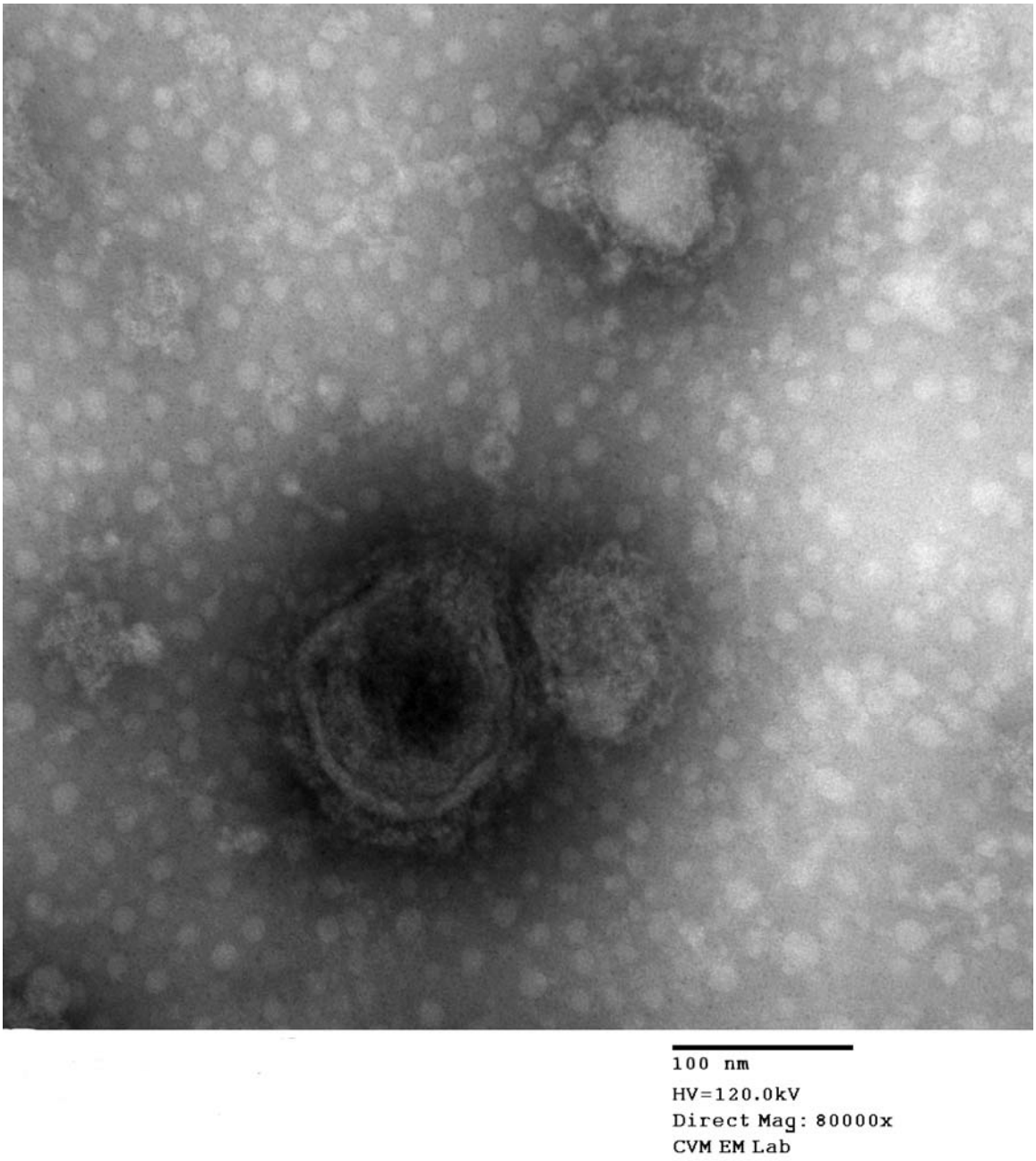

Figure 3.5: Negative stain image of LPAI H5N3. (A/Chicken/Texas/167280-4/02). Image was taken at the University of Georgia College of Veterinary Medicine Electron Microscopy Laboratory, Athens Georgia.

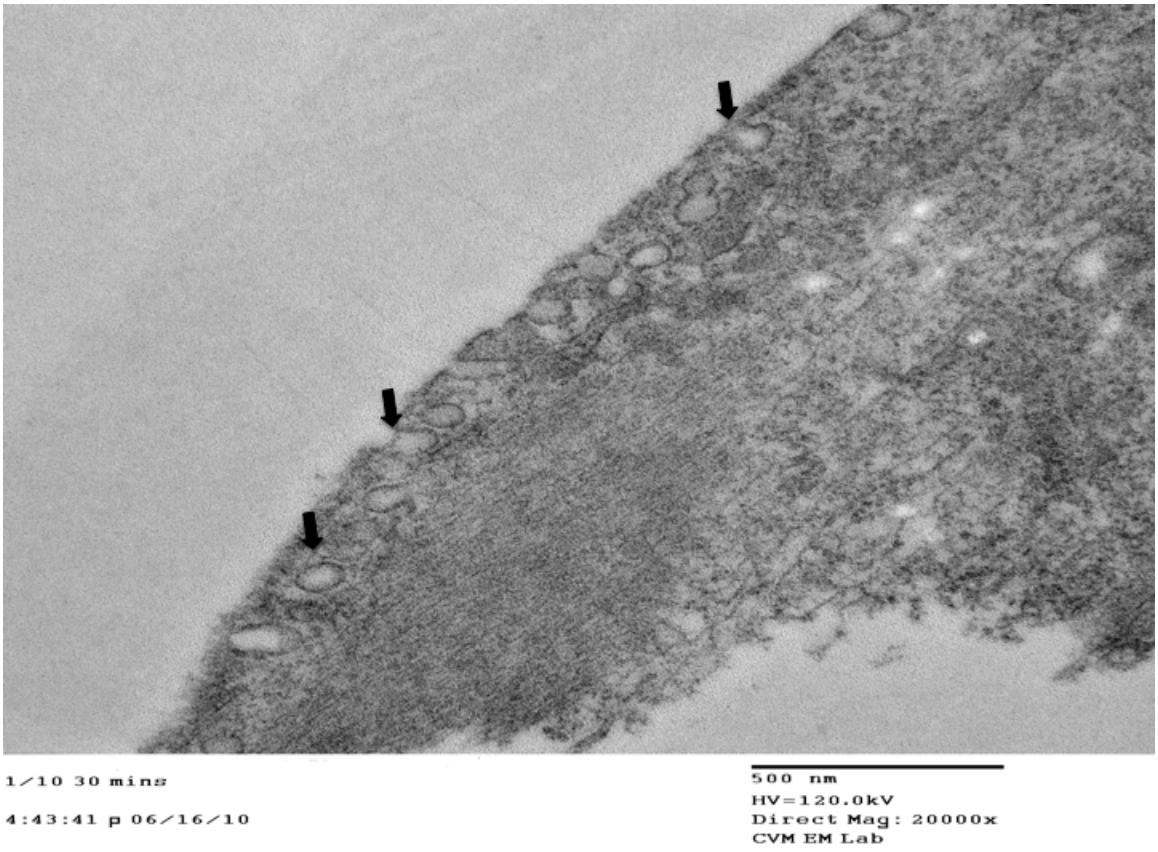

Figure 3.6: Ultra-structure of primary embryonic alligator fibroblast. Transmission electron microscopy image of alligator fibroblast cell infected with A/Chicken/Texas/167280-4/02 (H5N3) at an MOI of 0.5PFU/ml. The cells were fixed 30minutes post-infection with 2% (para)formaldehyde, 2% glutaraldehyde, 0.1M Cacodylate Buffer pH7.23. Arrows indicate possible virus entering the cell via plasma membrane.

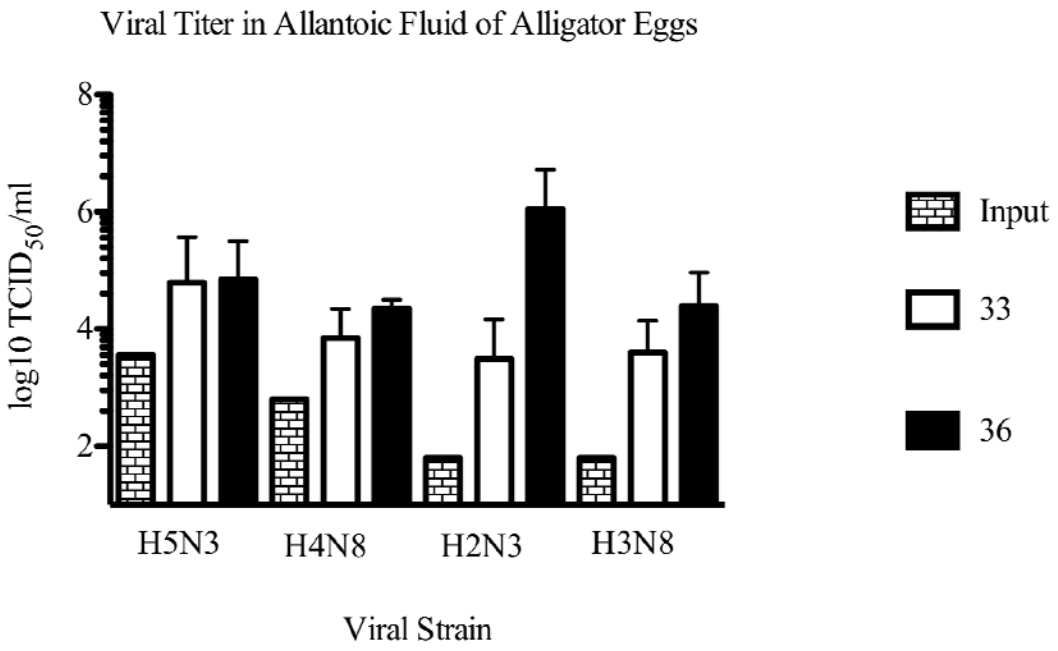

Figure 3.7: Mean viral titer levels in allantoic fluid from embryonated alligator eggs (n=5).

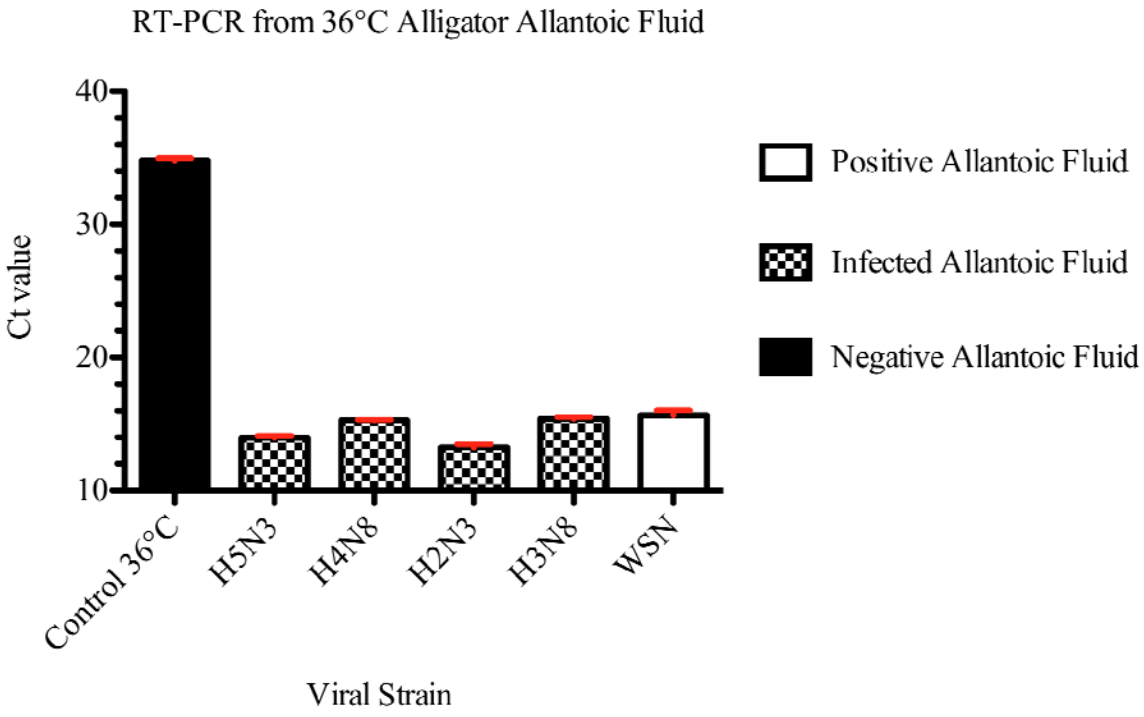

Figure 3.8: Real-time RT-PCR of M Gene from four LPAI viruses isolated from alligator allantoic fluid. Real-time RT-PCR results of mean C_t values from alligator allantoic fluid for all four LPAI. All infected samples were below the negative threshold of 34.79. Control (n=9) H5N3, H4N8, and, H3N8 (n=6) H2N3 (n=3), and WSN (n=9).

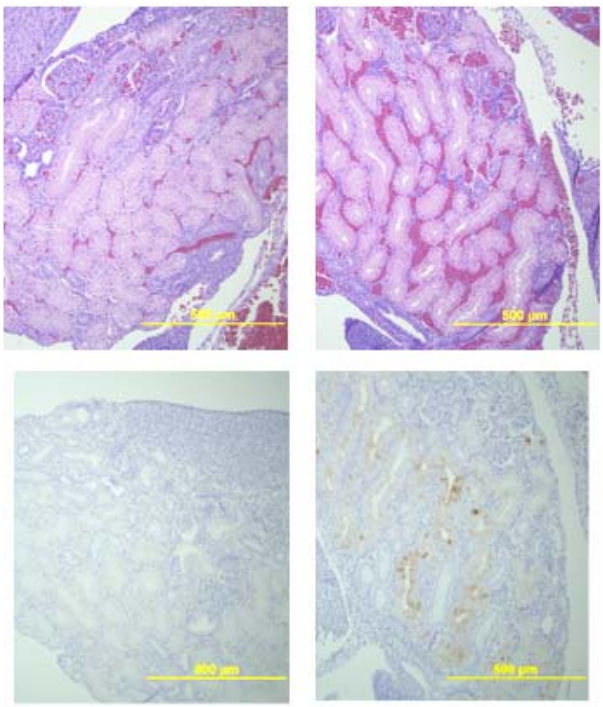

Figure 3.9: H&E and Immunohistochemical detection of control and H4N8 infected kidney. Sections of kidney from influenza infected and control alligator embryos. The top panels are photomicrographs of H&E stained sections. At the top left is a section from a control animal (33°C) that was inoculated with PBS and antibiotics. Top right image is from an embryo infected with H4N8 influenza (33°C) and is congested. The sections of kidney in the bottom panels were incubated with antibodies against influenza NP. The bottom left is from the same control animal shown in the top left H&E section and staining for NP is not observed. The bottom right panel is a serial section from the same H4N8 inoculated embryo shown in the H&E at the top right. Note the NP positive staining in the renal tubules.

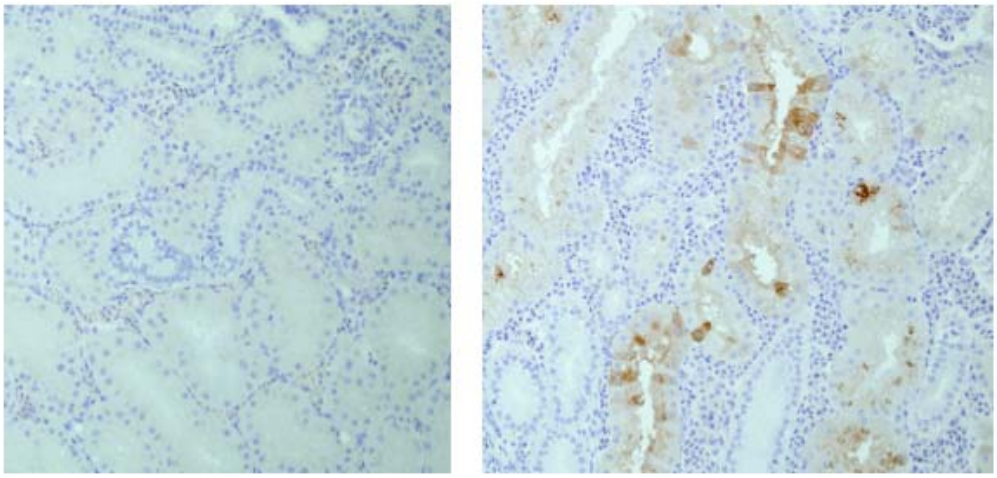

Figure 3.10: Immunohistochemical detection of LPAI H4N8 infected kidney. Higher magnification of the immunohistochemical detection of influenza infected and control alligators. At the left is a section of kidney from an embryo inoculated with PBS and antibiotics media (33°C) while the right panel image is from an embryo inoculated with H4N8 influenza virus. Note the positive staining in the renal tubules in the section from the influenza inoculated animal (x200).

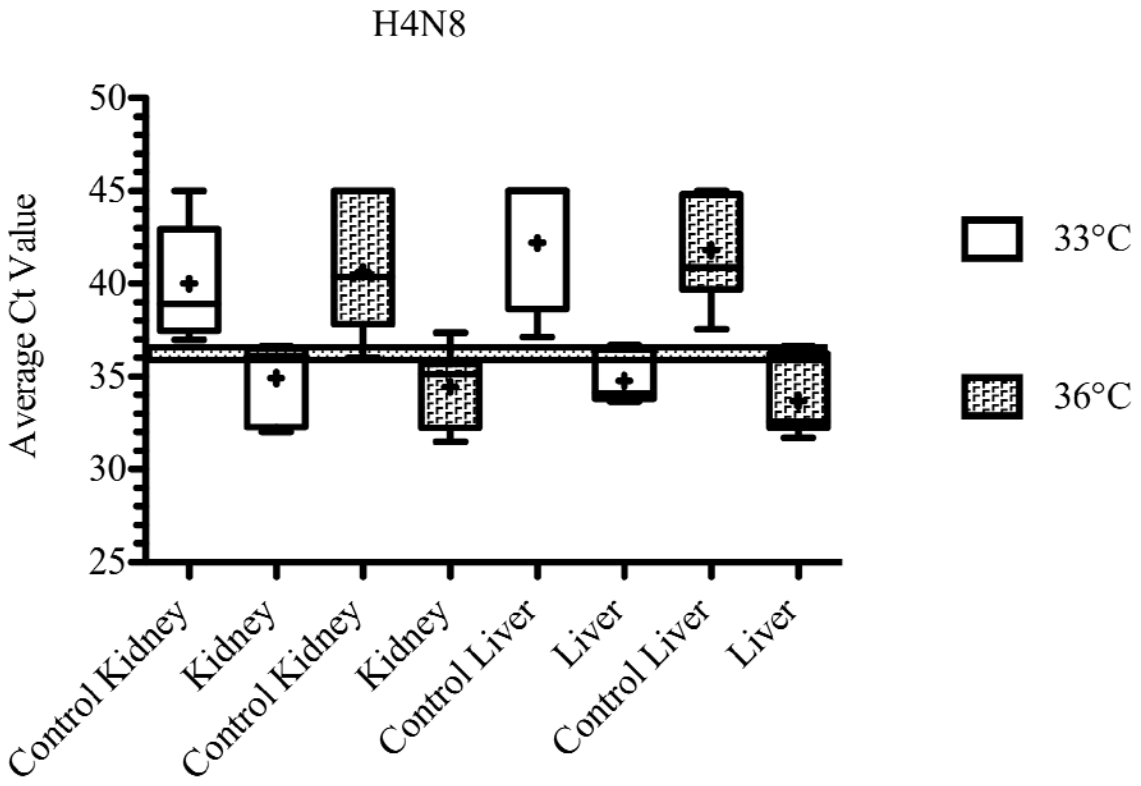

Figure 3.11: RT-PCR of M gene in H4N8 infected embryonic tissues. Real-time RT-PCR results of H4N8 Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected gator embryos. Both positive and negative controls performed as expected. All samples above a C_t value of 36.40 were classified as negative for the presence of M gene. All samples with a C_t value below 36.40 were classified as positive for influenza A genomic RNA. + indicates mean C_t value for all samples (n=9 except for 36°C control kidney and control liver n=12)

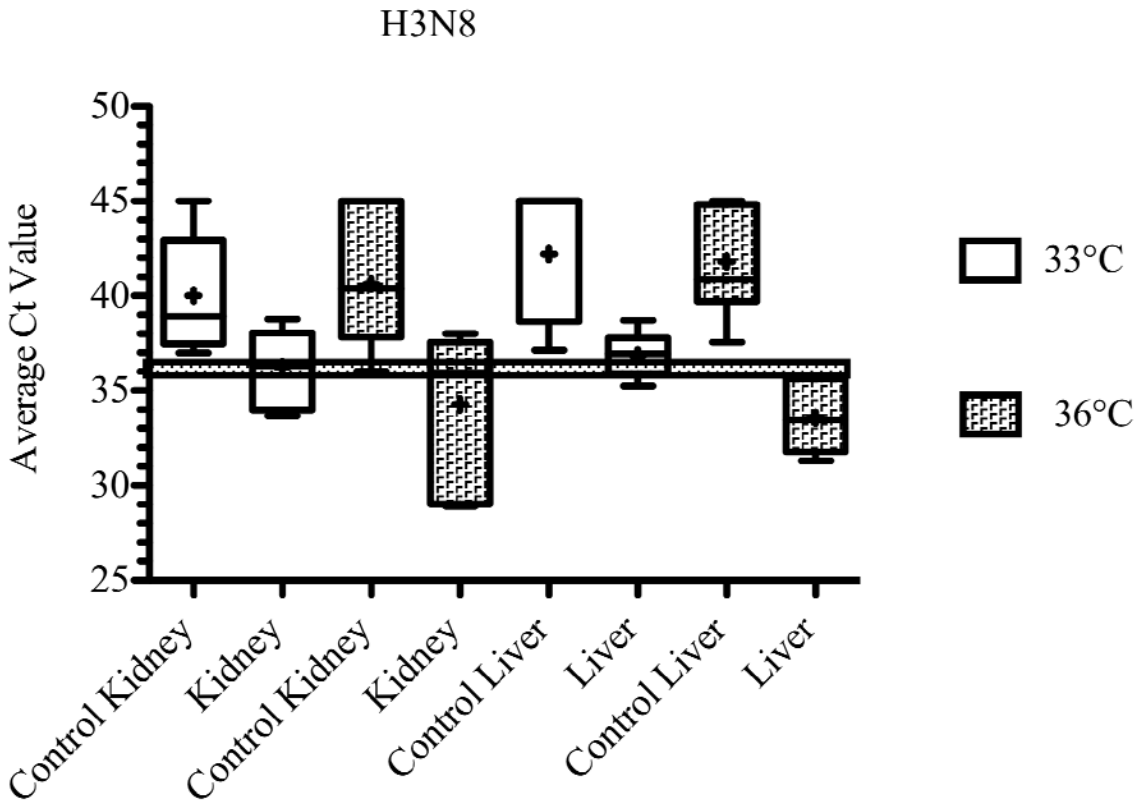

Figure 3.12: RT-PCR of M gene in H3N8 infected embryonic tissues. Real-time RT-PCR results of H3N8 Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected gator embryos. Both positive and negative controls performed as expected. All samples above a C_t value of 36.40 were classified as negative for the presence of M gene. All samples with a C_t value below 36.40 were classified as positive for influenza A genomic RNA. + indicates mean C_t value for all samples (n=9 except for 36°C control kidney and control liver n=12)

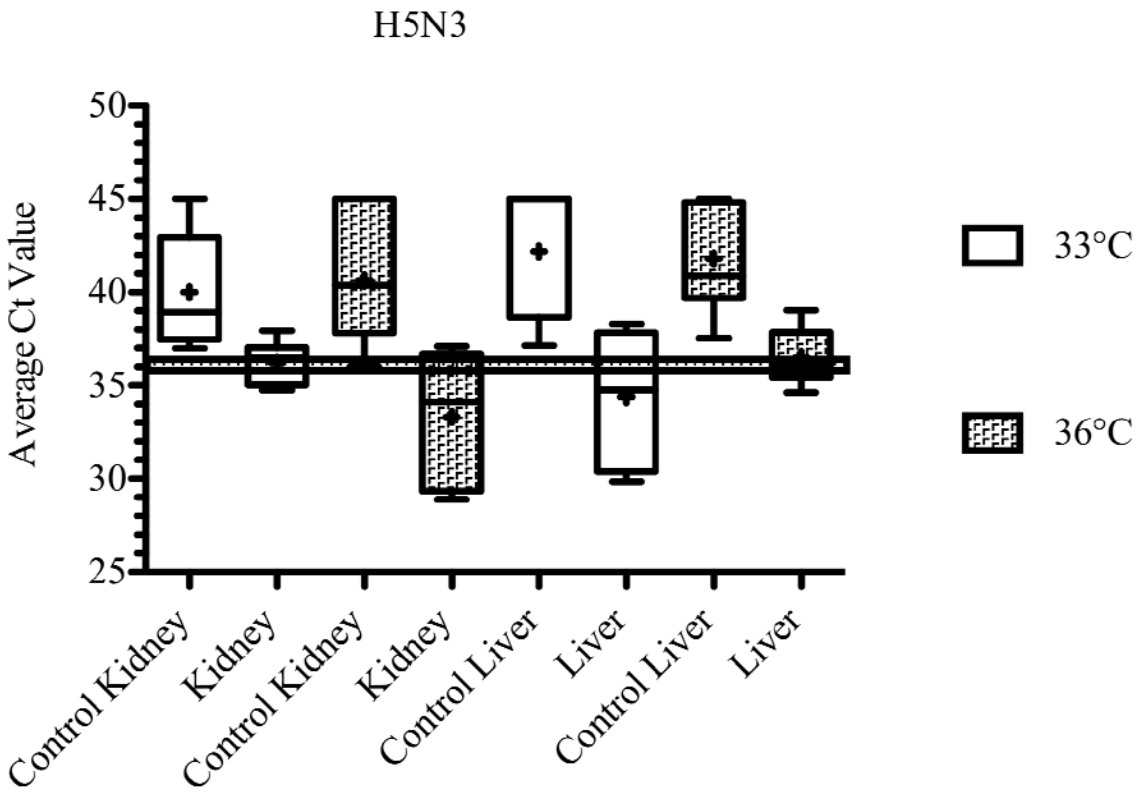

Figure 3.13: RT-PCR of M gene in H5N3 infected embryonic tissues. Real-time RT-PCR results of H5N3 Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected gator embryos. Both positive and negative controls performed as expected. All samples above a C_t value of 36.40 were classified as negative for the presence of M gene. All samples with a C_t value below 36.40 were classified as positive for influenza A genomic RNA. + indicates mean C_t value for all samples (n=9 except for 36°C control kidney and control liver n=12)

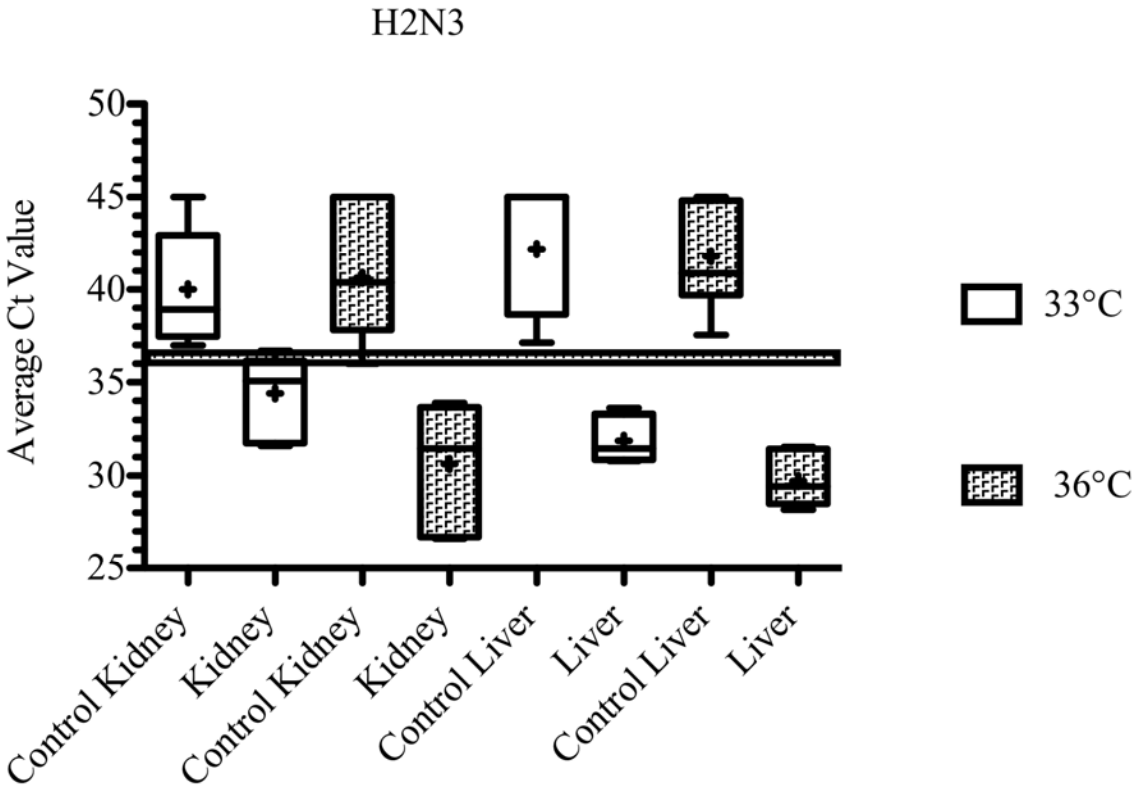

Figure 3.14: RT-PCR of M gene in H2N3 infected embryonic tissues. Real-time RT-PCR results of H2N3 Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected gator embryos. Both positive and negative controls performed as expected. All samples above a C_t value of 36.40 were classified as negative for the presence of M gene. All samples with a C_t value below 36.40 were classified as positive for influenza A genomic RNA. + indicates mean C_t value for all samples (n=9 except for 36°C control kidney and control liver n=12).

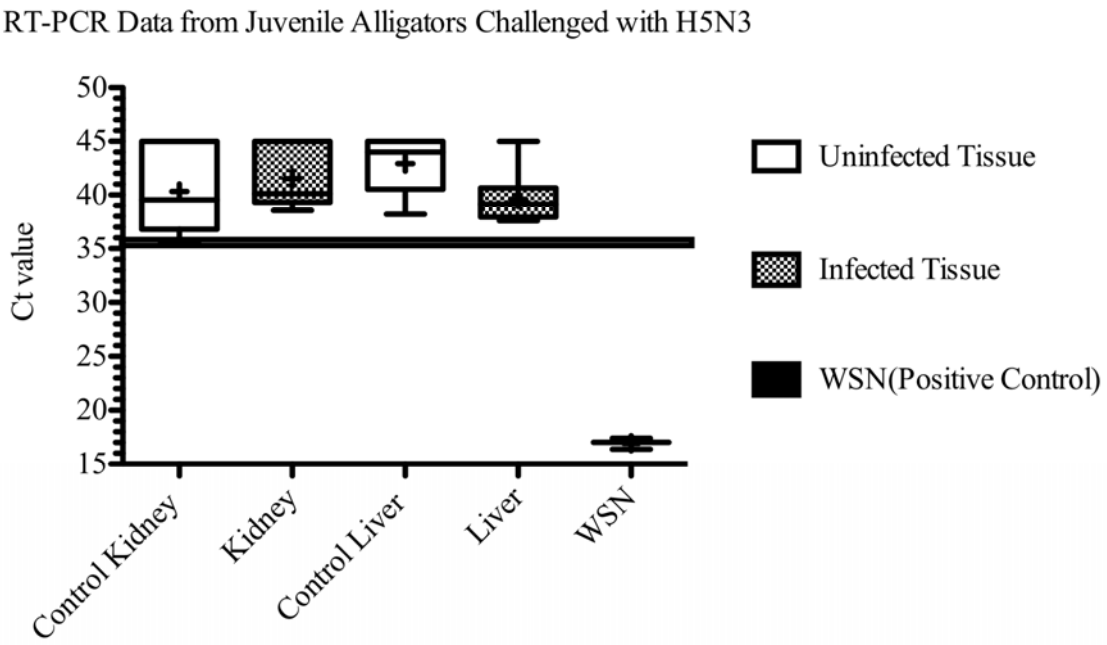

Figure 3.15: RT-PCR results from juvenile alligators challenged with H5N3. Real-time RT-PCR results of H5N3 Influenza A genomic RNA specific for M gene from kidney and liver samples of infected and uninfected juvenile alligators. Both positive and negative controls performed as expected. All samples above a C_t value of 36.40 were classified as negative for the presence of M gene. All samples with a C_t value below 36.40 were classified as positive for influenza A genomic RNA. + indicates mean C_t value for all samples (n=5 for infected tissue samples and n=2 control kidney and control liver samples) WSN influenza strain was used as a positive control strain.

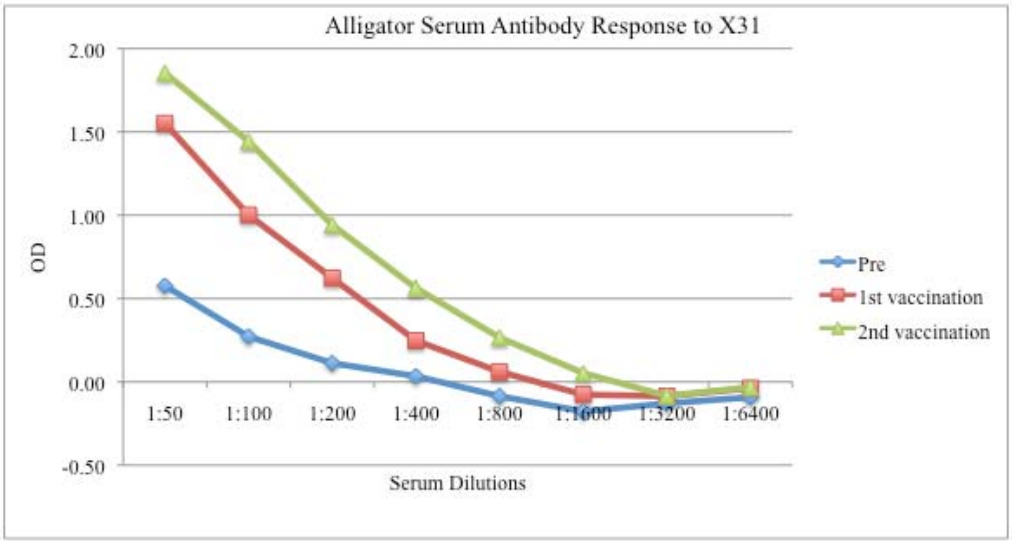

Figure 3.16: Alligator serum antibody response to H3N2. Immune response from serum isolated from a 2-3 year old alligator immunized with A/Puerto Rico/8/34. Serum from the same alligators collected at three time points: non-vaccinated (pre-immune), and 35 days post 1st vaccination 17 days post 2nd vaccination and assayed for antibody response against influenza A X31 strain (H3N2).

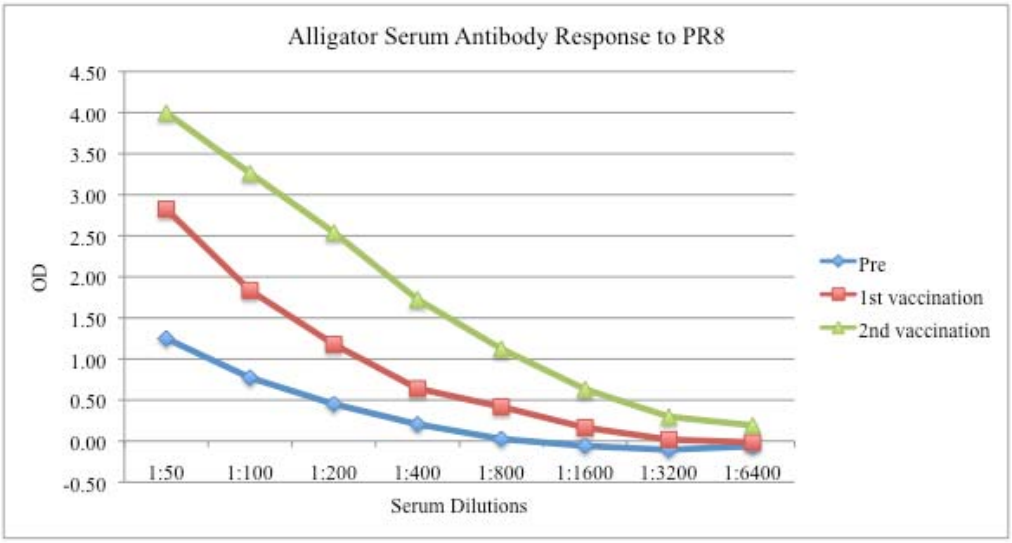

Figure 3.17: Alligator serum antibody response to H1N1. Immune response from serum isolated from a 2-3 year old alligator immunized with A/Puerto Rico/8/34. Serum from the same alligators collected at three time points: non-vaccinated (pre-immune), and 35 days post 1st vaccination 17 days post 2nd vaccination and assayed for antibody response against influenza A PR8 strain (H1N1).

CHAPTER 4

DISCUSSION

This study has demonstrated that four LPAI isolates are able to infect and replicate within a crocodilian system at temperatures much lower than typical wild-type avian influenza viruses would usually replicate. Wild-type avian influenza isolates generally replicate in the intestinal tract of avian species at approximately 41°C. Although much less efficient than at optimal temperatures, avian influenza has been shown to replicate at temperatures as low as 33°C (Massin et al. 2001, Massin et al. 2010).

Primary alligator fibroblast cells were positive for the presence of viral nucleoprotein after infection with each of the four viral strains used in these studies. Coinciding with previous research, all four viral strains appeared to produce a higher amount of NP protein at 37°C vs. 33°C. The presence of nucleoprotein inside of the nucleus demonstrates that alligator fibroblast cells are susceptible to infection with LPAI, and the virus has begun to produce both viral mRNA and new viral proteins. The results were inconclusive as to whether or not the virus was able to complete a replication cycle leading to production of progeny virus within the culture. However this could be due in part to three reasons: The first being that the virus was not cultured with exogenous trypsin, a necessary component for replication of LPAI because trypsin allows for the cleavage of HA to produce infectious virus. Secondly, since this is a primary cell line the cells may still be capable of producing an innate immune response, such as the production of interferons. Lastly, the cells may not be capable of producing undescribed virus-compatible cellular processes necessary for production of infectious virons. The presence

of NP does not necessarily constitute the production of progeny virus, but it does indicate that the process of viral replication has begun, including the synthesis of viral mRNA and proteins.

To address these issues, supernatant from cell culture infected with H5N3 was collected and assayed with a 72 hr viral titration plaque assay with MDCK cells in the presence of exogenous TPCK trypsin. A small portion of samples collected (2 out of 11 with all controls performing as expected) produced viral titers of $1\times10^{3.00}$ PFU/cell. However, most supernatant collected yielded no plaque forming units as measured by an avicel overlay plaque assay (Data not shown). Due to time constraints, we were unable to assay the cell cultures for the production of interferons. However, previous research has shown the production of interferons in primary chicken fibroblast cell lines when infected with vaccinia virus (Hornemann et al. 2003). The supernatant results imply that there may be some viable viral progeny being produced within the fibroblast culture; however, as previously stated the results are not encompassing enough to draw any clear conclusions concerning sustained viral replication. Although the results do clearly demonstrate the infection of alligator fibroblast cells, and the production of nucleoprotein at MOI's ranging from 0.0006 to 0.1PFU/cell at both 33°C and 37°C.

Along with antigen staining for NP protein fibroblast cells infected with H5N3 were also examined using transmission electron microscopy. Figure 3.13 shows an embryonic fibroblast cell from a culture infected with the LPAI H5N3. The arrows indicate possible virus entering the cell via the plasma membrane. No definitive evidence was presented upon examination of the EM images that would conclude with a high level of confidence that virus was seen entering, replicating within, or exiting the cell. However, the cells were infected with an MOI of 0.5 PFU/cell, which in all likelihood was too low of an MOI to observe virons using EM analysis.

With that said, the infected cells viewed from both the 6 and 12 hr post infection cultures showed a higher level of vacuolization within the cells than either the 30-minute or negative controls cultures. Upon observation the 12 hr cultures had a higher level of cell death, apparent by the level of cells and cellular debris present in the supernatant visible under light microscopy, when compared to the negative control wells at the same time points. When the cells were viewed using EM the nuclei appeared to be under going apoptosis (Data not shown). While EM did not clearly demonstrate viral replication, viral replication is one possible cause of these phenomena and further investigation is warranted.

While the primary alligator cell lines offer a model for investigating viral replication under carefully controlled conditions, and indicate that alligator cells are susceptible to infection with LPAI strains the results obtained from these studies are not suitable for determining whether hatched alligators could serve as viable host for viral replication. This question was investigated using a two-pronged approach. First, embryonated alligator eggs were inoculated with four LPAI viral strains, and secondly a small group a juvenile alligators were infected with the LPAI H5N3 strain.

The results from the juvenile challenge study suggest that alligators are not susceptible to viral replication. However, this conclusion is a premature as it is unclear if non-cold adapted (wild-type) avian influenza viruses can replicate at temperatures below 33°C. The data does not preclude the possibility that the AIVs could infect alligators as virus infection can occur at sub-optimal temperatures as seen with four LAPI viruses in Figure 3.7. Temperature is still a key component driving viral replication (Massin et al. 2010), and since the ambient temperature of the alligator housing never exceeded 30° C it is possible that the alligator's body temperature was insufficient to support viral replication.

The embryonated alligator eggs present an entirely different scenario. All four viral strains replicated within the embryo with all four strains infecting both the liver and kidneys. Moreover, all virus-inoculated eggs had high virus titers in the allantoic fluid. Out of the four viral strains chosen for this experiment the H2N3 strain replicated to the highest titer in embryonated eggs, but only at 36°C where the mean viral titer in allantoic fluid was $10^{6.04}$ (±0.67) $TCID_{50}$/ml. At 33°C the H5N3 viral strain replicated best with a mean viral titer of $10^{4.79}$ (±0.77) $TCID_{50}$/ml vs. $10^{3.49}$ (±0.67) $TCID_{50}$/ml in H2N3. The H4N8 and the H3N8 both had mean viral titer in between these values at both temperatures. The H2N3 strain indicated the highest-level temperature sensitivity in regards to viral replication amongst the four strains. In contrast the other three stains indicated no statistical change in viral replication. In four viral strains the virus titers were several logs greater than the input titers indicating virus replication in all four strains at both temperatures.

The H2N3 strain is one of only three sub-types of influenza viruses that have established themselves in humans (Ma et al. 2007). This subtype was also responsible for the 1957 flu pandemic in humans and has recently established itself in swine (Xu et al. 2010). The H4N8 strain was isolated within one year and within the same geographical location as an influenza strain previously identified in the literature as being isolated from a species a crocodilian (Davis and Spackman 2008). The H5N3 was a LPAI strain considered to be a progenitor to a highly pathogenic avian influenza (HPAI) (Lee et al. 2004). This may explain why temperature was less of a factor in the replication of this strain when compared to the others, as one characteristic of the HPAI is the ability to replicate at temperatures as low as 33°C (Hatta et al. 2007). The H3N8 and H4N8 were both isolated near the location where the alligators for this study were collected.

The results from the embryonated alligator eggs demonstrate that influenza can replicate to high levels within the embryonated eggs (up to $10^{7.75}$ $TCID_{50}$/ml), but not to these same levels in the embryo itself. Embryonic tissues, liver and kidney, of infected eggs were positive for both genomic RNA and NP antigen (Figures 3.9-3.14). This suggests that the virus infected and began replication within the tissue, producing both genomic RNA and viral proteins, but infectious virus was not measurable above the level of detection by cell-based ELISA ($10^{2.00}TCID_{50}$/ml). The most common sites of infection for avian species infected with AIV are the lower respiratory tract and the gastrointestinal tract. Therefore, we hypothesized that viral infection and replication could occur in similar tissues within alligators, however, it is not uncommon in reptiles to see viral infection and replication within the liver and kidney (Gans 1969, Just et al. 2001).

The real-time RT-PCR data correlate well with the viral titers level within the embryonated alligator eggs for each strain. The H2N3 strain had the highest viral titers of each of the four strains at 36°C and likewise had the highest mean levels of M gene genomic RNA in both liver and kidney at the same temperature. The other three strains showed production of genomic RNA but not to the same level as the H2N3 strain. Similar trends were seen in the allantoic fluid where temperature played a diminished role in viral replication in the other three strains when compared to H2N3. These RT-PCR results, in conjunction with the NP staining in the tissues and detection of high viral titers within allantoic fluid clearly establishes production of infectious H2N3 viral progeny within a crocodilian system, and shows that 36°C is the optimal temperature (of the temperatures) for viral replication with embryonated alligator eggs.

Nonetheless, the RT-PCR data in conjunction with IHC and viral titration also suggest that the other three strains investigated can infect various tissues and replicate within the

crocodilian system under sub-optimal conditions for viral replication (i.e. temperatures less than 36°C). This observation may have more importance than the H2N3 strain data because this temperature range would be more ideal for alligators found in the wild. The infection of and production of viral RNA and proteins at these low temperatures within a crocodilian system suggest the ability for this virus to possibly replicate within American alligators at temperatures well within their natural metabolic range (Gans 1969).

Although the evidence presented did not demonstrate the replication of live infectious progeny virus within juvenile alligators, there are several reasons that could have produced this outcome. One possible explanation is that the MOI used for infection was too low. It is also possible that the alligator immune response in conjunction with a low MOI and temperature created an environment not suitable for virus replication. Alligators do possess cell-mediated immunity through the production of both NK cells and T lymphocytes, and recent evidence has reported the phagocytic activity of B-cells in reptiles (Zimmerman 2010). Alligator serum does exhibit some anti-viral activity against a wide range of enveloped viruses including WNV, Herpes simplex, and HIV (Merchant 2005). The role of a innate immune response is also supported by data suggesting that alligators do not begin to produce a humoral immune response (HIR) until after two months post-hatching(Gans 1969).

Alligators, unlike mammals, only produce two types immunoglobin IgM and IgY, (Gardner and Oberdörster 2006), and current literature has demonstrated the efficacy of IgY antibody produced in avian species and its protection against LPAI H5N1(Nguyen et al. 2010). Since IgY is considered the predecessor to IgG it may offer some protection in alligator from influenza A (Warr et al. 1995).Temperature in alligators, like other reptiles, plays a role in most physiological processes including the immune response. Studies have indicated a decrease or

absence of production of alligator immunoglobin production at low temperatures, and an increased rate of production at temperatures within optimal metabolic range (Origgi 2002).

The results seen in the embryonated alligator eggs are not unlike those characterized in avian and other reptile species. Vaccinia virus has been shown to replicate, indicated by the presence of Guarnieri bodies, within the chorioallantoic membrane (CAM) of two species of turtle eggs, *Pseudemys troostii* and *Chrysemys pict dorsalis* (Harris 1945). Avian eggs inoculated LPAI are unable to demonstrate replication within the embryo as well, and instead virus is only present within the (CAM). Unlike the HPAI, which can replicate within the embryo itself (Capua et al. 2002). Alligator eggs appear to follow this same model, although the presence of both the IHC staining in the liver and kidneys and the positive RT-PCR results lends credibility to the hypothesis under more optimal conditions influenza A virus may replicate not only within the CAM and but also within the tissues. The ability of AIV to replicate in the CAM of alligator eggs is not surprising. The eggs of alligators and birds share several similarities and with the exception of some distinct physiological and morphological differences such as the fusion of the chorion and allantoic membranes to each other, along with fusion to eggshell itself in alligator eggs. Along with the aforementioned differences alligator eggs also have an increase in both protein composition and viscosity in the allantoic fluid when compared to avian eggs. (Ferguson 1982). The membranes in both eggs serve similar functions and both species are believed to have derived from a common ancestor. One could hypothesize that the cell type within the CAM of the alligator egg more closely resembles that of the avian cells types present inside the CAM of avian eggs creating a more conducive environment for viral replication even under less than optimal conditions. However, we are unaware of any studies that have characterized cell types present within CAM of alligator eggs.

CHAPTER 5

CONCLUSION

This study has demonstrated that alligators are susceptible to LPAI infection, and that these viruses can replicate within embryonated alligator eggs. This was clearly demonstrated by the levels of infectious virus present in allantoic fluid. Along with viral titer in allantoic fluid, IHC results indicated that alligator embryos exhibited a degree of viral replication in both the liver and kidney. Documented by the presence of NP protein, genomic viral RNA, necrosis, congestion, and lesions in the liver and kidney of alligator embryos. However it remains unclear if the necrosis, congestion, and lesions were due directly to the virus or if the replication of the virus within egg altered normal physiological process, which in turn led to these responses.

Even though infection and replication were not documented in alligators older than one year, under tested conditions, it is important to continue to monitor and investigate crocodilian populations for the possible presence of influenza virus. Cold adaptive viruses have been shown to replicate at temperatures much lower that wild-type isolates(Massin et al. 2001). This in conjunction with evidence suggesting alligator immune response is closely linked to temperature makes monitoring and understanding alligator response to both wild-type and cold adaptive AIV paramount to determining the ecological role crocodilians may play in AIV replication and transmission.

While crocodilians have existed for approximately 240 million years these species are still susceptible to a variety of diseases and environmental changes. WNV has been documented as causing skin lesions in older alligators, and death in the case of younger

crocodilians. Other species of crocodilians are nearly extinct in the wild and must be closely monitored to ensure their continued survival. The Chinese alligator (*Alligator sinensis*) is an endangered species that in 2002 had less than 130 animals in wild populations (Thorbjarnarsona 2002) and thus could be highly vulnerable to extinction due to a viral outbreak. The importance of ensuring that populations like the Chinese alligator are not impacted harshly by influenza A is just one of several reasons for understanding the role that crocodilians may play in the transmission of influenza A. Currently, no data exists on the susceptibility of crocodilians to HPAI viruses. Given the proximity of this endangered crocodilian population to outbreaks of both LPAI and HPAI, along with data presented from this and previous studies on crocodilian viruses it would be prudent to investigate the susceptibility of crocodilians to HPAI.

Further experiments are needed to determine how LPAI replicates within embryonated alligator eggs. Additionally, the mechanism(s) within the embryos that offered protection from the influenza A viruses replicating within the eggs is not well understood and would benefit from further investigation. Also, we have shown the alligators can produce an antibody response to multiple strains of AIV when vaccinated with a single strain indicating the production of cross-reactive influenza A specific antibodies. Current literature has begun to focus on alligator immune responses due to their strong level of immunity to bacterial pathogens (Merchant et al. 2005, Merchant et al. 2009). Studies have shown alligator serum possessing anti-viral activity, but the question remains how much that response is passed on to the offspring during development. Therefore, serum samples from wild populations, along with farmed populations, should be screened to determine if there is currently or has been environmental exposure to AIV.

More experimentation should be conducted on the cell lines and the biological response of both the cells and the animals from exposure to both LPAI and HPAI isolates. This primary cell line offers a method to better characterize the innate immune response process in crocodilians when exposed to infection. Lastly, this alligator cell line would allow us to better distinguish which AIV may have the most impact on both wild and captive crocodilian populations.

In summary we have shown the ability of four LPAI to infect and begin replication within a primary embryonic alligator fibroblast-like cell line. Along with demonstrating the ability of LPAI to replicate at sub-optimal temperatures inside of 41-51 day old embryonated alligator eggs, and infect and produce viral NP and viral genomic RNA in the liver and kidneys of those same alligator embryos. This study has established a need to further investigate the role of crocodilian species in the ecology of avian influenza viruses.

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