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Lymphohistiocytic Proliferative Syndrome of Alligators (*Alligator mississippiensis*): a cutaneous manifestation of West Nile virus

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LYMPHOHISTIOCYTIC PROLIFERATIVE SYNDROME OF ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*): A CUTANEOUS MANIFESTATION OF WEST NILE VIRUS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences through
the Department of Veterinary Clinical Sciences

by
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B.S., Louisiana State University, 1998
D.V.M., Louisiana State University, 2001
May 2007

To the gators.

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ABSTRACT

In 1999, there were reports of a new type of lesion in the hides of captive reared alligators from Florida. Similar lesions were first reported from alligator hides in Louisiana in 2001; however, it wasn't until 2002 that small epizootics became apparent. In 2002, the Louisiana Department of Wildlife and Fisheries began a collaborative effort with the Louisiana State University School of Veterinary Medicine (LSU SVM) to help elucidate the etiology of "PIX" disease, later renamed Lymphohistiocytic Proliferative Syndrome of Alligators (LPSA).

Preliminary work concluded that LPSA was a systemic disease affecting multiple tissues. Based on the results of this preliminary study, particularly the histopathologic evaluation of LPSA tissues, a viral etiology was established as the top differential for LPSA. Further work revealed that LPSA positive alligators were 476 (95% CI: 79.6, 2845.2) times more likely to be seropositive for WNV than LPSA negative alligators. At that point it was also becoming clear that the occurrence of LPSA matched the occurrence of WNV in alligator farms. Another project was performed to further elucidate the association between WNV and LPSA based on results of WNV serology, WNV RT-PCR, and histopathologic evaluation of animals with (treatment) and without (control) LPSA lesions. Results from this study revealed that in the treatment group, 97.5% (95% CI: 92.7-102.3 %) of the LPSA skin lesions (TxA) were positive for WNV via RT-PCR. Of the skins within the treatment group that had no LPSA lesions (TxB), 8% (95% CI: 0-16.9%) were positive for WNV. In the control group, all of the skin samples (CxS) were negative for WNV. All alligators in TxA were significantly ($p=0.07^{20}$) more likely to have RT-PCR WNV positive skin than those in CxS, and TxB ($p=0.08^{16}$). There was no significant difference in the recovery of WNV from the skins of alligators from TxB and CxS ($p=0.24$).

The results of this work support the theory that LPSA is a cutaneous manifestation of chronic WNV exposure/infection in captive reared alligators. Therefore the epidemiology of LPSA follows the epidemiology of WNV and prevention, surveillance and control methods used for WNV should effectively decrease the occurrence of LPSA.

INTRODUCTION

Modern day crocodilians date back over 65 million years to the Mesozoic era, yet they have been able to survive with relatively small evolutionary changes. Their biology, physiology, and anatomy are very different from other reptiles. Unfortunately, these evolutionary marvels are now starting to suffer from anthropogenic influences on their environment, and many are now threatened or near extinction. Of the 23 species of crocodilians found worldwide, 13 can be found on the red list of endangered species (IUCN 2004). Fortunately, there are now international conservation programs dedicated to the preservation of these species.

In the United States, the American alligator (*Alligator mississippiensis*) is the most common and best-known crocodilian. The only other species of native crocodilian found in the United States, the American crocodile (*Crocodylus acutus*), is a highly vulnerable species that has a limited range in southern Florida. The American alligator was also at critical levels during the 1960's, but a federal conservation program designed to protect wild populations through farming and ranching has brought their population numbers back to sustainability. In Louisiana alone, it is estimated that there are over one million animals. The first type of operations consisted of farms. Breeding pairs of alligators were kept in outdoor enclosed areas where they could mate and nest (Figure 1). The eggs were then collected and incubated. The farming operations later developed into a ranching operation in which eggs were harvested from the wild and incubated in private facilities (Figure 2). The majority of the hatchlings in this system are raised for their hide and meat; however, to help maintain the wild populations, 14% of the hatchlings are eventually returned to the wild. Today, Louisiana is the world's primary producer of alligators; however, captive rearing operations can also be found in Florida, Texas, Georgia, and other southern states alligators naturally inhabit. In Louisiana, the majority of the operations are ranches.

In 1999, there were reports of a new type of lesions in the hides of captive reared alligators from Florida. These skin lesions were described as 1mm pit scars on tanned alligator hides (Dickson 2001, Cardeilhac 2001). These scars were termed “PIX” disease because the lesions, according to the tanners, appeared as if made with an ice pick. Original work performed by Cardeilhac et al. (Cardeilhac et al. 2001) described the lesions as lymphocytic infiltrations and granulomas with a weakened cornified layer. They also observed fungal hyphae in some of the



Figure 1. Outdoors enclosure used for farming Morelet’s crocodiles in Mexico.



Figure 2. Buildings used for alligator ranching in Louisiana.

lesions and were able to obtain fungal growth from one sample. The fungus was identified as *Hortaea werneckii*, and it was believed to be at least in part the presumptive agent for “PIX” disease (Cardeilhac 2001).

Similar histologic lesions were first reported from alligator hides in Louisiana in 2001; however, it wasn't until 2002 that small epizootics became apparent. In 2002, the Louisiana Department of Wildlife and Fisheries (LDWF) began a collaborative effort with the Louisiana State University School of Veterinary Medicine (LSU SVM) to help elucidate the etiology of "PIX" disease. In addition to performing research dealing with "PIX" disease, the LSU SVM also provided clinical veterinary services to the Louisiana Alligator Industry (LAI). Early on during these collaborative efforts the authors re-classified "PIX" disease as Lymphohistiocytic Proliferative Syndrome of Alligators (LPSA). This classification was used because it provided a histopathologic description of the original skin lesions. The work presented here describes the research and clinical work performed between 2002 and 2007 to define the epidemiology of LPSA in alligators from Louisiana alligator ranches.

Biological Hypotheses

1. LPSA is an internal/systemic disease.
2. The etiology of LPSA is infectious because affected animals are generally found in the same pen or building.
3. The histopathologic lesions associated with LPSA are consistent with viral disease.
4. LPSA is a clinical sign associated with West Nile virus (WNV) infection in alligators.
5. The appearance of LPSA in farmed alligators in Louisiana coincided with the appearance of WNV in Louisiana.

LITERATURE REVIEW

Because of their relative age, crocodylians represent an important group of vertebrates. These animals have survived for millions of years utilizing many unique evolutionary features. Most of our knowledge regarding these animals has come through research; however, the majority of the research is related to evolutionary biology, physiology, taxonomy and behavior. To date, there have been few examples of research evaluating the health of these animals. The purpose of this literature review is to provide a concise review of our current knowledge regarding these animals.

Louisiana Alligator Industry

The LAI is regulated by the LDWF, and has its roots in the Rockefeller Refuge in Grand Chenier, Louisiana. During the 1960s the population of wild alligators in Louisiana was estimated at less than 100,000 (LFAC 2007). Because of the decreasing population size, a moratorium on alligator hunting and regulation of alligator harvest were implemented in an attempt to re-establish the alligator populations. As part of those efforts, an alligator farming and ranching program was established in order to promote alligators as a natural renewable resource in the state. This program would help conserve alligator habitat as well as promote the preservation of wild alligators. An alligator ranching program was established in 1986. Under this program, the LDWF granted egg harvest permits to licensed alligator ranchers in the state. There are currently 59 alligator ranches in the state and collectively they produce approximately 390,000 alligators every year. This market has an annual value of approximately 30 million dollars. The alligator ranching system works via the sale of egg harvest permits by LDWF for collection of alligator eggs from private and public lands. Egg collection usually takes place between June and August. Eggs numbers can range from 20 to 60 per nest. Once the eggs are collected, they are taken to a private facility where the eggs are incubated in designated buildings

or incubators. The eggs collected from the wild are at different stages of incubation, so once at the alligator ranch it may take anywhere from a few weeks to months for the eggs to hatch depending on the time of collection. The total incubation period ranges from 40 to 90 days. Incubation temperature will determine the sex ratio of the alligators between the 7th and 35th day of incubation. Temperatures of 30°C (86°F) or below will yield a higher proportion of females while temperatures >34°C (93°F) will yield a higher proportion of males (Grenard 1991). The majority of alligator ranchers do not attempt to select for a specific sex. Once the alligators are hatched, they are placed in indoor buildings where they are raised until the time of slaughter. Alligators are usually raised in 18 to 20 inches of water. The water depth may be adjusted depending on the size of the alligators and the condition of their skin. A wooden table is sometimes placed in the pens and used as a feeding surface, while others place the food directly on the water. Dry commercial feeds ranging in protein content from 45 to 56% are readily available. The majority of the facilities will feed a commercial diet exclusively, while others mix in fish, chicken, or nutria meat. Within 10 to 12 months, alligators can be raised to a market size of 36 inches. At that point the ranchers begin the slaughter of the alligators in order to sell the hide and meat. The alligator hides are sold to dealers and tanners for further processing. The LAI is the largest supplier of alligator hides in the world. Louisiana alligator hides eventually end up in high end retail products such as watch bands, shoes, purses, and other fashion items. These products have the benefit of coming from a natural renewable resource that is environmentally friendly and helps contribute to the wild populations of alligators. In addition to the hide, alligator meat is sold to processing plants and distributed to restaurants, supermarkets, and specialty stores.

The conservation aspect of the LAI comes from the 14% of alligators at each facility (based on the yearly egg harvest) that are destined for release back into the wild. The percent of

alligators released is based on an expected survival of 10 to 20% of the hatchlings under natural conditions, and amounts to approximately 55,000 alligators being released back to the wild every year (LFAC 2007). Alligators being released to the wild are identified using toe tags and a tail notch. This program has been extremely successful, and has served as an important model for other crocodylian species around the world. Today, the population of wild alligators is estimated at well over 1 million animals, a 90% increase from the 1960s estimates.

Taxonomy

The taxonomic identification of crocodylians is variable, with some authors arguing over what should be classified as a species or a sub-species. Tables 1 to 3 list the generally accepted 23 crocodylian species by their common name, scientific name, and geographic distribution. These lists are not meant to be absolute, but rather a general guideline. Countries such as Australia, India, Mexico, Papua New Guinea, South Africa, and the United States maintain intensive production operations for various species.

Anatomy and Physiology

One of the most commonly asked questions' regarding a crocodylian is how to distinguish a crocodile from an alligator. The first major difference is that they belong to two different families. There are currently three distinct families of crocodylians: Alligatoridae (Table 1) (alligators and caimans), Gavialidae (Table 2) (gharials or gavials), and Crocodylidae (Table 3) (the crocodiles). Geographical location can also help in the identification of some species. Alligators are thought to tolerate colder temperatures and live at higher latitudes, while crocodiles and caimans are less cold resistant and live in warmer areas (Huchzermeyer 2003). However, there are some anatomical features that will be most useful in differentiating alligators from crocodiles. Alligators and caimans have a broad u-shaped snout, while crocodiles have a more narrow v-shaped snout. This comparison can be made when looking at the dorsal aspect of

Table 1. Taxonomy of the Family Alligatoridae

| Common Name | Scientific Name | Geographical Distribution |
|--------------------------|-----------------------------------|----------------------------------|
| American alligator | <i>Alligator mississippiensis</i> | Southeast United States |
| Chinese alligator | <i>Alligator sinensis</i> | Eastern China |
| Spectacled/Common caiman | <i>Caiman crocodilus</i> | Central and South America |
| Broad-snouted caiman | <i>Caiman latirostris</i> | South America |
| Jacare caiman | <i>Caiman yacare</i> | South America |
| Black caiman | <i>Melanosuchus niger</i> | South America |
| Cuvier's dwarf caiman | <i>Paleosuchus palpebrosus</i> | South America |

Table 2. Taxonomy of the Family Gavialidae.

| Common Name | Scientific Name | Geographical Distribution |
|-----------------------------|----------------------------|----------------------------------|
| Indian gharial/True gharial | <i>Gavialis gangeticus</i> | Indian subcontinent |

the head (Figure 3). A more obvious distinction can be made when looking at their mouth from the side. Alligators and caimans have notches in the maxilla that fit the mandibular teeth. Therefore, they have no mandibular teeth visible if observed from the side with their mouth closed. On the other hand, the fourth mandibular tooth of crocodiles is readily apparent when looking at the profile of the animal (Figure 4). On close inspection of the integument, one can also look for the presence of integumentary sensing organs (ISO's) or dome pressure receptors (DPR's). These receptors are clear to gray pits that are present on the skin of crocodilians, but their location varies with species. Their function is not completely understood, but they may play a role as mechanoreceptors in prey detection or even as chemoreceptors aiding

Table 3. Taxonomy of the Family Crocodylidae

| Common Name | Scientific Name | Geographical Distribution |
|--|--------------------------------|-----------------------------------|
| American crocodile | <i>Crocodylus acutus</i> | North, Central, and South America |
| Slender-snouted crocodile | <i>Crocodylus cataphractus</i> | Africa |
| Orinoco crocodile | <i>Crocodylus intermedius</i> | South America |
| Fresh water crocodile/Johnston's crocodile | <i>Crocodylus johnstoni</i> | Australia |
| Philippine crocodile | <i>Crocodylus mindorensis</i> | Philippines |
| Morelet's crocodile | <i>Crocodylus moreletii</i> | Central America |
| Nile crocodile | <i>Crocodylus niloticus</i> | Africa and Madagascar |
| New Guinea crocodile | <i>Crocodylus novaeguineae</i> | Papua New Guinea and Irian Java |
| Mugger crocodile/Swamp crocodile | <i>Crocodylus palustris</i> | Indian subcontinent |
| Saltwater/Estuarine crocodile | <i>Crocodylus porosus</i> | Australia and Southeast Asia |
| Cuban crocodile | <i>Crocodylus rhombifer</i> | Cuba |
| Siamese crocodile | <i>Crocodylus siamensis</i> | South East Asia |
| African dwarf crocodile | <i>Osteolaemus tetraspis</i> | Africa |
| False gharial | <i>Tomistoma schlegelii</i> | South East Asia |

in detection of salinity levels (Soares 2002, Britton 2005). Alligators and caimans only have ISO's on the lateral aspect of the mandible (Fig. 5). Crocodiles and gharials have ISO's throughout the body, most noticeably on the ventral scales (Fig. 6). Their presence can be used to differentiate the skins from the two main groups in the leather market. An additional feature that could be used for differentiation is the presence or absence of salt glands, which are absent in the tongue of alligators and caimans but well developed in crocodiles and gharials.

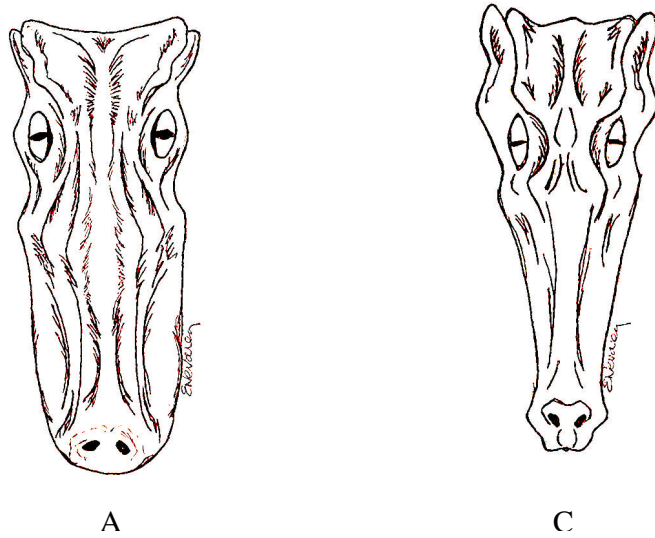


Figure 3. Dorsal view comparison of caiman and alligator (A) versus crocodile (C).



Figure 4. Lateral view comparison of caiman and alligator (A) versus crocodile (C).

One of the most interesting anatomical features of crocodylians is the palatal or gular valve. There is some discrepancy as to the name of this structure and its two components, but I will attempt to describe them based on their anatomical location. Crocodylians have a true hard palate that ends in a soft palate caudally. This soft palate has a ventral flap that is referred to as the velum palati. The velum palati is the dorsal component of the palatal valve. Its second and ventral component is the gular fold. This structure projects in a craniodorsal direction from the



Figure 5. Integumentary sensing organs (gray dots) on the lateral mandible and maxilla of an alligator.

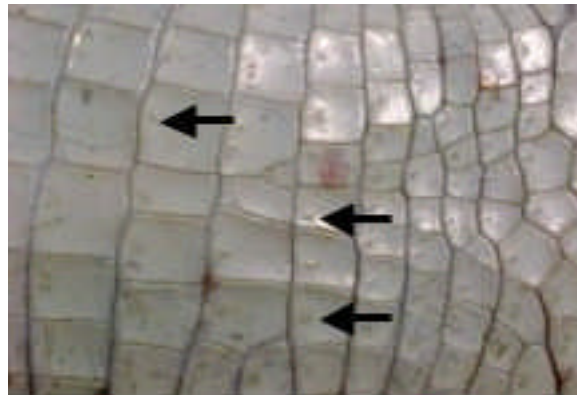


Figure 6. Integumentary sensing organs (arrows) on the ventral skin of a crocodile.

base of the tongue and has a cartilaginous base to it that is part of the larynx. Together, the velum palati and the gular fold form what is known as the palatal or gular valve (Fig. 7). The function of this valve is to seal the pharyngeal cavity while under water to prevent aspiration, allowing these animals to catch prey under water. Crocodylians also have control of their nares, and are able to open and close them as needed to prevent water influx.

The respiratory system of crocodylians is comprised of a pair of well- developed lungs. Respiration in these animals is an active process, and is aided by the intercostal muscles and the septum post hepaticum. The septum post hepaticum is analogous to a mammalian diaphragm, and creates a partial separation of the thoracic and abdominal viscera. There are also a number of membranous connections that separate the lungs and the liver and an intricate mesentery

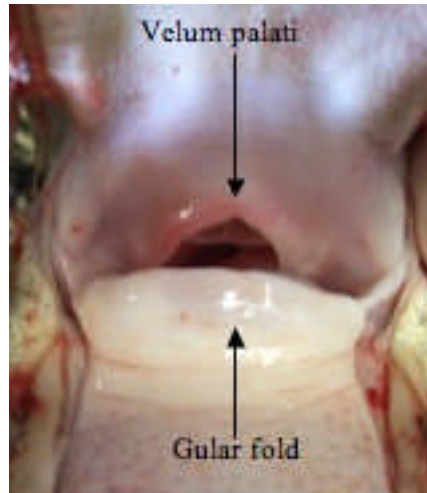


Figure 7. Picture of the palatal valve with its two components, the velum palati dorsally and the gular fold ventrally.

system that encompasses the gastrointestinal tract and viscera. All these modifications may be necessary for allowing the changes in pressures that occur during diving.

The cardiovascular system of crocodilians is also unique. Crocodilians have a four-chambered heart, as opposed to the standard three-chambered heart found in other reptiles and amphibians. The circulation of blood through the crocodilian heart is like that in the heart of mammals; however, there are two separate anastomoses that enable these animals to shunt blood during times of breath holding (e.g., diving). The foramen of Panizza is an anastomosis that is located at the base of the heart between the left and right aortic arches. This foramen allows for venous admixture, which is essential during periods of diving in order to conserve oxygen (Fleming 2001). During diving (e.g., breath holding), pulmonary hypertension occurs. This in turn creates increased pressure in the pulmonary artery and the right ventricle, forcing deoxygenated blood through the foramen of Panizza and into the left side of the aorta for distribution to the body. This mechanism allows for the conservation of oxygen, and supplies oxygenated blood to those organs that require it the most. As a consequence, some crocodilian present in some species as a vessel connecting the two aortic arches (Huchzermeyer 2003).

Other anatomical features include the presence of submandibular and paracloacal glands. The hard dorsal scales are comprised of osteoderms, which are bony plates located in the dermis. They also have a smaller gastric compartment distal to the stomach that is a gizzard-like structure in which rocks and other materials may be found. It, however, does not appear as developed as the ventriculus in a bird. Their intestines have a thick wall and can have well developed diffuse aggregates of lymphoid tissues analogous to Peyer's patches. Crocodilians have no urinary bladder, but can hold large amounts of urine and water in their colon. A gall bladder is present. Sexing can be performed by palpation. Males have a phallus, which can be palpated and extracted from the cloaca. Females have a well- developed clitoris that can be confused with a phallus. Internally, they have paired gonads that are in close association with the ventral surface of the kidneys.

The body temperature, heart rate and respiration will vary with species and environmental factors. Environmental temperature, season of the year, and age represent some of the factors that can influence physiologic data.

Husbandry

Environmental Considerations

The environmental considerations for crocodilians will vary with the scenario in which you are working. In captive rearing operations, facilities are aimed at producing large number of animals in the most efficient manner possible. In a zoo or other educational institution, the facilities are aimed at exhibiting the animals in the most accurate artificial environment possible. Regardless, both scenarios should concentrate on having clean water, appropriate diet, and enough space to accommodate the growth of the animals. As with most exotic animals, the challenge is to mimic their natural environment as closely as possible in captivity.

Enclosure Size

There are no specific recommendations for enclosure size for crocodylians in captivity. The size of the enclosure will largely depend on the species of crocodylian and their purpose in captivity. You should have a general understanding of their biology and natural behavior in order to design an appropriate enclosure for display (e.g., zoo or aquarium). If in doubt, you should contact an institution housing the species for advice on what has worked or not worked for them. There are, however, some general guidelines for the commercial production of American alligators (*A. mississippiensis*). The recommendations for maintaining alligators in a commercial operation are as follows: One square foot per alligator up to 24 inches in length (snout to tip of tail), three square feet per alligator for those between 25 and 48 inches in length, and after that add an additional square foot of space for every 6 inches in body length beyond 48 inches (Elsley 2004). These are considered to be recommendations for the maximum stocking rate for alligators in commercial operations.

For a zoo or educational facility, the recommendation should be made to make the exhibit as big as possible. First, consider the species of interest. Some species grow larger than others and may be more territorial, which would require a larger enclosure. You must also consider how many animals will be kept in the enclosure and the gender ratio of animals in the enclosure. Males can become quite aggressive during the reproductive season, and keeping them separated should be a consideration if enough space is not available. Exhibits can be maintained outdoors, indoors, or using a combination of both. Outdoor exhibits will likely mimic the natural environment more closely, but also present more challenges for maintaining hygiene of the water and environment and exposure to diseases. Geographical location will also play a role in the creation of outdoor exhibits, as not all species of crocodylians can tolerate cold weather. Finally, some species can generate large burrows, and may escape an exhibit that is not secured.

Temperature and Humidity

The temperature and humidity requirements for crocodylians in captivity vary with species. Once again, it is important to have an understanding of their biology and natural history to be able to duplicate their natural environment. The provision of both circadian variations in the light cycle and environmental temperature to mimic the natural environment should be made. Unfortunately, this is not the case in many commercial operations, where they are maintained at a fairly constant temperature and humidity in order to obtain faster growth. From a health standpoint, this may allow infectious organisms that typically affect mammals to also affect these reptiles. As the animals are maintained at higher temperatures, approaching those of mammals, new diseases may adapt to infecting reptile hosts. In an enclosure, the water temperature can be maintained via heating elements contained within the concrete slab or in line water heaters if using a re-circulating system. The water temperature must also be maintained during the refilling of the pen or enclosure to avoid a drastic temperature change.

Lighting

Lighting requirements for reptiles are still a controversial subject. As a general rule, full spectrum lighting possessing adequate ultraviolet B radiation (UVB) is recommended for herbivorous and omnivorous reptiles. Ultraviolet light is essential for the synthesis of vitamin D₃, specifically its active form 1, 25 dihydroxyvitamin D, which is essential for the metabolism of calcium and phosphorus. A deficiency of vitamin D₃ can lead to inappropriate calcium absorption, which in turn creates a metabolic imbalance (e.g., metabolic bone disease). Metabolic bone disease, and more specifically secondary nutritional hyperparathyroidism, is recognized in many reptile species that are not provided UVB and/or calcium. Carnivorous reptiles may also benefit from UVB, but are thought to obtain enough vitamin D₃ and calcium from their prey. The UVB requirement for crocodylians is unknown, but as true carnivores it

might be expected that they can thrive with minimal exposure to UVB. It is a common practice at alligator ranches to raise these animals in darkness with no source of UVB or a normal light cycle. Most animals will grow well under these conditions, and some have even been grown to adulthood without signs of metabolic diseases. However, the author has also observed evidence of metabolic bone disease in a subset of captive American alligators being fed a commercial diet and with no exposure to ultraviolet light. In these cases, we must also consider the possibility that the commercial diet may be deficient in calcium. Anecdotal stories from alligator ranches claim that weak, anorectic animals appear to improve after being exposed to sunlight over a period of time. Further research is needed to determine the UVB requirements of crocodylians, and the potential benefits of exposure to UVB. Natural unfiltered sunlight is the best source of UVB, but various artificial sources are also available.

Substrate

The two main substrates used in a crocodylian exhibit are water and soil/sand. The species, age, and feeding habits must be taken into account in order to avoid selecting a substrate that may be accidentally ingested. It is also important to prevent the public from throwing coins and trash into exhibits, as this may represent a source of toxicity and foreign body impactions. In commercial operations, a smooth covering is applied to the concrete in order to preserve the quality of the hide. Either an epoxy coating or plastic liners are routinely used as substrate.

Nutrition

Crocodylians are true carnivores, as evidenced by their short intestinal length and cutting/tearing teeth. As such, they will require a high protein diet that is low in fiber. Their feeding habits in the wild will vary with age and food availability. The diet of juvenile animals often is comprised of small invertebrates, amphibians, and other reptiles. As they grow, they will pursue larger sized prey of the same type and add fish and birds to their diet. As adults, they will start

preying on small mammals too. There has been some study into the nutritional requirements of alligators (Staton et al. 1990) (Coulson et al. 1987). Different feeding schemes were also evaluated in the early 1990s (Coulson et al. 1992). In captivity, there are various commercial feeds available for alligators. These are extruded pellet diets that aim to meet the complete nutritional requirements of an alligator. These diets are generally comprised of 45%, 47%, or 56% protein, less than 11% fat, and fiber content less than 3% (Burriss 2005). Over time these diets have been refined. They are widely used in commercial ranching operations, but may prove too expensive and/or inappropriate for the long term feeding of alligators. A variety of whole prey feeds, such as chicken, nutria and fish, may also be recommended. If using nutria, you must be sure that it was not killed using lead shot, as this can lead to lead toxicosis in alligators (Camus et al. 1998). If feeding fish that has been frozen, you must also supplement the diet with thiamine to prevent thiamine deficiency.

Preventive Medicine

Quarantine

All newly acquired crocodylians should be quarantined before being introduced into a facility. A detailed history from the facility providing the animals should also be obtained in order to gather information related to previous disease history. It is important to always work with reputable individuals or institutions. Wild caught animals rarely come with much history. Crocodylians should be quarantined for a minimum of 60 to 90 days. Quarantine should always be done in a building that is separate from the main facility. During this time, the animals can be examined for any sign of illness, and diagnostic tests (e.g., complete blood count, chemistry, West Nile virus (WNV) antibodies) performed to assess the overall health status of the animals. If they originated from an area where WNV is prevalent, it may be advisable to test for antibodies to determine exposure to WNV.

Unfortunately, a true quarantine process can not be done in many cases because of limited facilities, time or money. Quarantine can also be a real challenge for commercial operations where you may be dealing with tens of thousands of animals. Nonetheless, zoological institutions should always attempt to quarantine crocodilians.

Routine Exams

Routine physical exams should be performed on crocodilians at zoological institutions as part of their preventive health program. As animals get larger in size, this activity may become more hazardous. Chemical immobilization can be used, if needed, in order to perform necessary examinations. In commercial operations, the skin of a subset of animals will be examined at some interval before the time of slaughter. These instances present an opportunity for veterinarians to examine the animals in this type of operations. Other than these, routine physical exams are not commonly performed in crocodilians in commercial operations.

Biosecurity

Biosecurity is an essential part of disease prevention in any animal facility, and crocodilians are no exception. A sanitation station should be placed at the entrance of each building. These stations should contain a footbath and a hand washing station. The presence of these stations should remind individuals of the importance of biosecurity, and minimize the transfer of diseases between exhibits or buildings. A brush should be provided at each footbath to clean the boots/shoes thoroughly. The solution for the bath can be made of sodium hypochlorite or other commercial disinfectant, preferably with virucidal activity. Footbaths should be changed daily or as they are dirtied since organic material can deactivate disinfectants. The footbath should always be used before and after entering the building. A hand washing station should also be provided. If a water source is available, then a sink and hand soap should be provided. Alternatively, you can provide a waterless hand sanitizer product or exam gloves.

In addition to the sanitation station, separate tools for working in each building should be provided. This will also prevent the transfer of diseases via nets, brooms, rakes, or other cleaning supplies. The buildings themselves must also be maintained free of pests and cleaned thoroughly when possible. In commercial operations, it is common practice to empty and disinfect the buildings after the slaughter period and before introducing new animals. Finally, the water the animals are housed in should be cleaned regularly. Poor water quality often leads to the development of health problems in these animals. Regular complete changes of the water and/or the addition of filtration can be used to maintain the quality of the water.

Handling and Restraint

Manual restraint of crocodilians is required for examining, medicating, and transporting/relocating crocodilians. The size and species of crocodilian will determine the most appropriate restraint method to use. Always work with someone who is familiar with the behavior and restraint of a particular species. Alligators and caimans are usually thought of as being less aggressive than crocodiles, but this may not always be true. All sizes and species should be handled carefully, considering both the safety of the individuals restraining the animal and the animal itself. Crocodilians less than 3 feet in length (snout to tip of tail) may be handled by one or two individuals. Those between 3 and 6 feet in length should be handled by at least two or three individuals. Those longer than 6 feet in length will require at least four to five individuals for safe restraint. There are a number of restraint tools, such as pole snares, nets, squeeze cages and traps, which can be used to restrain crocodilians. The head, tail, and limbs must be immobilized and kept under control to effectively immobilize a crocodilian. Once the animal is captured, the mouth should be secured with strong tape or a rope. Albino and leucistic animals can have a more sensitive skin than those that are normally pigmented, and should therefore be handled more carefully. Restraining a crocodilian is a stressful procedure, so it is

important to minimize the amount of contact time spent with the animal. Chemical restraint should be considered for aggressive or overly fractious animals.

History and Physical Examination

Signs of illness in captive crocodylians usually begin as non-specific in nature. Anorexia, lethargy and death may be the first indication that something is wrong in a collection of animals or commercial operation. A change in the behavior of the animals may also be observed. A visit to the facility should be performed during feeding time to avoid additional stress to the animals. A thorough review of the husbandry practices should be evaluated. A thorough history should include questions about the number of animals, source, age, most recent introduction, quarantine practices, diet, general husbandry practices, frequency of feeding, water quality parameters, clinical signs, time since first signs were observed, recent changes in management techniques, and whether any treatments have been provided. If working in a commercial operation, a subset of animals should be collected for diagnostics and necropsy. In addition to obtaining routine samples, tissues should be frozen for possible bacterial, fungal, or viral cultures. If working in a zoological institution, you may not be able to sacrifice live animals, but you should obtain diagnostic samples from those with and without clinical signs. Necropsies should be performed in all dead animals. Live animals should be examined closely.

Once the animal is properly restrained, you can perform a thorough physical exam. You must, however, be aware of the location of the head and tail at all times for your own safety. The physical exam is performed as in any other species. An oral examination can be performed if a speculum is inserted into the mouth before securing it with tape or a rope. A plastic PVC pipe or piece of wood can be used for this purpose (Fig. 8). Examine the eyes for evidence of discharge, and determine if the third eyelid is functioning properly (Fig. 9). Examine the skin for any evidence of trauma or dermatitis, two common findings in commercial operations. Palpate the

extremities and joints for any evidence of swelling. Joint swelling is a common sequellae to mycoplasmosis or trauma. Examine the musculature of neck, pelvic region and tail. Poor body condition may be reflected in these areas. In commercial operations, it is important to examine the skin on the ventrum. Many disease manifestations will be noted on the ventral skin. It is



Figure 8. A speculum inserted in the mouth of an alligator allows for visualization of the oropharyngeal cavity. This may also be used as a protective guard while performing endoscopy or retrieving foreign bodies from the intestinal tract.



Figure 9. Conjunctivitis with involvement of the third eyelid in a Morelet's crocodile.

also common to find tooth marks, scratches, and lacerations. Finally, examine the vent and cloaca for any abnormalities. If working in a commercial operation, examine multiple animals to determine if an observation is associated with disease in multiple animals. If working in a zoological institution and any findings are suspected to be infectious in origin, you should examine other animals in the exhibit. As with any species you need to be aware of what is normal in order to recognize clinical signs of disease.

Diagnostic Testing

Diagnostic testing in crocodylians should follow the same standard protocols advocated with other species. One of the limitations associated with some of the diagnostic tests available for crocodylians is that they are not validated or are based on a loose set of reference ranges. It is important to always inquire about the specifics of a test in order to make an accurate interpretation of the results. Published literature and the experience of other colleagues is an invaluable part of interpreting diagnostics in crocodylians.

Venipuncture

There are various sites for venipuncture in crocodylians. The ventral coccygeal vein can be accessed from either the ventral or lateral aspect of the tail (Fig 10). This vessel lays ventral to the vertebral processes and on midline with the vertebrae. A second alternative is the



Figure 10. Venipuncture being performed from the ventral coccygeal vein using a lateral approach.

supravertebral sinus located on midline at the junction of the head and the neck (Fig. 11). You must be careful not to insert the needle past the spine, as it may be possible to damage the spinal cord. A third site for venipuncture is the lateral occipital sinus, located lateral to the supravertebral sinus (Wilhite and Nevarez 2004). This site can be approached from the dorsal aspect of the neck, and is surrounded by muscles, decreasing the risk of contact with the spinal



Figure 11. Venipuncture being performed from the supravertebral sinus in an alligator.

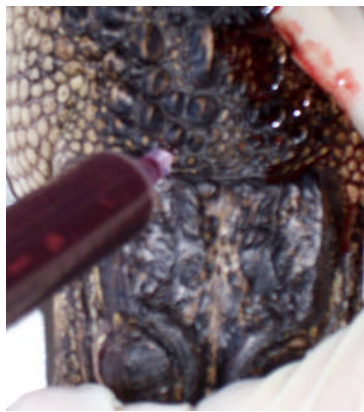


Figure 12. Venipuncture being performed from the lateral occipital sinus in an alligator. Notice that it is farther away from midline, eliminating the risk of damaging the spinal cord.

cord (Fig. 12). A 3cc syringe and a 22 gauge needle can be used for blood collection. Lithium heparin and EDTA tubes can be used for chemistries and complete blood counts, respectively. If collecting serum, the tubes may have to sit for at least 45 to 60 minutes before centrifugation to allow proper separation of the serum from the red blood cells.

Clinical Pathology

As with other species, complete blood counts (CBC's) and chemistry panels are a fundamental part of diagnostics in crocodilians. These tests can help us assess the overall health status of an animal. A CBC is generally used to measure whether an animal has a leukocytosis (e.g., stress or inflammation) or leukopenia. Plasma chemistry panels can provide insight into the physiologic status of an animal. Measurement of the packed cell volume (PCV) and total

solids/ total proteins of an animal are also important to provide insight into the erythron, hydration status, or possible inflammatory response (e.g., hyperglobulinemia) of a patient. Fine needle aspirates, impression smears and fluid analysis are useful diagnostic tools in crocodilian medicine. Urinalysis is not a practical test in crocodilians due to the absence of a urinary bladder and functional loop of Henle, and the fact that they will likely release the urine product in the water.

A CBC can be done using a hemacytometer with the Unopette Eosinophil Determination for Manual Methods stain (Becton Dickinson and Company, Franklin Lakes, NJ 07417-1885). A blood smear stained with diff quick (Quik-dip stain, Mercedes Medical Physician and Laboratory Products, 7490 Commerce Ct. Sarasota, FL 34342) is also needed to complete your total estimated white blood cell count and obtain the cell differential count. Interpretation of CBC's from crocodilians can be challenging at first, if you are not familiar with the morphology of their white blood cells. In order to familiarize yourself with these cells, spend some time scanning the blood smear to become familiar with all the different types of cells and their appearances. Alternatively, the samples can be submitted to a commercial laboratory that is comfortable interpreting crocodilian CBC's.

Imaging

Most imaging modalities (e.g., radiographs, Computed Topography scan, Magnetic Resonance Imaging, and ultrasound) can be used for crocodilian imaging, as long as there is a general understanding of their anatomy. This will be crucial for the interpretation of diagnostic images. Knowledge of the anatomy will also help locate organs during ultrasound examination. Radiographs can be used to locate foreign bodies as well as diagnose fractures. Contrast studies can also be performed. Barium sulfate or iohexol can be administered at 5-20 ml/kg PO to improve the quality of the images. Iohexol can be diluted with water at a 1:1 ratio (Carpenter et

al. 2001). Depending on the size of the animal and the site of interest, some imaging procedures may be performed with the animal under manual restraint only. However chemical restraint may be required in some instances.

Stress and Immunosuppression

Stress has been defined as “a physiological answer to a perceived threat that includes, but is not restricted to, increased adrenal secretion” (Rooney and Guillette 2001). Stress can also be any event that challenges homeostasis. The response of the body to that event is complex and involves more than an adrenal response. The autonomic nervous system, the hypothalamic adrenal axis, neuropeptides, neurotransmitters, and neuroimmunological mediators all play a role in the response of the immune system to stress (Dohms and Metz 1991). Measuring stress and immunosuppression is a challenge in veterinary medicine. There are no specific tests available to provide a clinical measure of stress. Because of this, we generally concentrate on identifying a combination of physiologic changes that give us an idea of what is involved in a stress response. The stress response in crocodilians has been examined in relation to restraint, long term corticosterone implants, cold shock, and stocking densities (Rooney and Guillette 2001, Lance and Elsey 1999, Morici et al. 1997, Lance and Elsey 1999a, Elsey et al. 1990). Lance et al. provides an overview of the physiology and endocrinology of stress in crocodilians (Lance et al. 2001). Catecholamines, glucocorticoids, glucose, and lactate have been implicated in the stress response of crocodilians. Changes in the white blood cells have also been implicated with immunosuppression and the stress response (Lance and Elsey 1999, Morici et al. 1997, Lance and Elsey 1999a, Lance et al. 2001). There is enough evidence to suggest that stress plays an important role in the physiology of crocodilians, and it may indeed predispose them to illness. Overcrowding, handling, excessive noise, diet changes, and temperature irregularities should all

be considered as predisposing or confounding factors of disease. All these factors must be considered in the history of a clinical case and at the moment of making recommendations.

An example of how stress can play a role in disease susceptibility can be observed in a case seen by the author. A commercial alligator facility had a chronic history of dermatitis. The alligators with the most severe lesions were consistently located on one end of the building while those at the other end were unaffected or only mildly affected. The location of the severely affected animals was consistent in all buildings. One building had no affected animals. After obtaining a thorough history and visiting the operation, a possible explanation for the occurrence of disease was found. All the buildings with affected animals had PVC pipes on the inside walls that delivered water to each individual pen. A strong water stream fell from the pipes down into the water and the strength of the stream decreased from one entrance of the building (inflow) to the other entrance (outflow). The strength of the water stream was considerable near the inflow. The building with no affected animals did not have any source of falling water into the pens. Water quality, temperature, and diet were the same in affected and non-affected buildings. The most affected animals happened to be in the pens at the inflow side of the building with the number of affected animals decreasing as you approached the outflow side of the building. This constant flow of water was creating a constant movement of the water surface and consequently stimulating the alligators via the ISO's. Once this watering system was changed, the cases of dermatitis decreased and no new cases were reported. Although other factors such as water quality may have contributed to the dermatitis, the change in the watering system decreased the progression and occurrence of the disease. Other stressors for captive reared alligators include construction and drastic changes in water temperature. This reiterates the importance of addressing the environment as well as the animals when working in commercial operations.

Bacterial Diseases

Most bacterial infections in captive crocodilians are probably opportunistic in nature. Poor water quality, trauma, and stress are some of the factors that contribute to bacterial infections in crocodilians. Crocodilians in their natural environment seem to cope well with most bacterial infections. This may be in part to reports of antibacterial properties in serum and tissues of crocodilians (Merchant et al. 2003, Shaharabany et al. 1999). This remains a controversial topic and some are investigating it further. It may also mislead people into thinking that crocodilians are not susceptible to bacterial infections. It is true that they appear to tolerate trauma and other lesions that would be fatal in many species, but they are still capable of succumbing to an array of microorganisms, including bacteria. In fact, a number of bacterial infections have been reported in crocodilians and septicemias are thought to be a frequent finding. Septicemias are definitively diagnosed post-mortem and can be associated with a wide number of bacteria, many of them generally associated with the intestinal microflora. There is an abundance of information about bacteria recovered from American alligators (Gorden et al. 1979, Shotts et al. 1972, Millichamp et al. 1983, Jacobson 1984, Flandry et al. 1989, Novak and Seigel 1986, Wallace et al. 1966, Mainster et al. 1972, Brown et al. 2001, Jacobson et al. 1984, Clippinger et al. 2000, Barnett and Cardeilhac 1995, Newton 1992, Bounds and Normand 1991, Russell and Herman 1970, Brown et al. 2001a) (Table 4) and African dwarf crocodiles (*Osteolaemus tetraspis*) (Huchzermeyer et al. 2000). Some bacteria (e.g., *Aeromonas* sp.) are commonly isolated from crocodilians, while others (e.g., *Erysipelothrix* sp. and *Clostridium* sp.) are not as common.

Salmonellosis

The importance of *Salmonella* sp. arises more from its zoonotic potential rather than its ability to cause disease in crocodilians, although it has been associated with mortalities in Nile

crocodile (*C. niloticus*) hatchlings (Huchzermeyer et al. 1994). In most commercial operations, the meat is sold as a by-product of hide production. Various species of *Salmonella* have been isolated from the meat of crocodiles from commercial operations, and may represent a potential source of infection for humans (Barnett and Cardeilhac 1995, Russell and Herman 1970, Millan et al. 1997, Madsen 1996, Madsen 1993, Manolis et al. 1991, Rickard et al. 1995). Petting zoos at zoological institutions that include crocodilians may also serve as a potential zoonotic source of infection for humans. Although the zoonotic potential for this organism does exist, there are no well- documented cases of human infections originating from crocodilians.

Chlamydiosis

Chlamydiosis has also been reported in crocodilians. There is a report of an isolate closely resembling *Chlamydophila psittaci* that was obtained from the livers of Nile crocodiles in Zimbabwe (Huchzermeyer et al. 1994). This infection is thought to have an acute course, and was characterized by hepatitis and heavy mortalities in hatchlings. A chronic form characterized by conjunctivitis has also been reported and may be more common. There are also other reports of *Chlamydia* sp. isolates in cases of mycoplasmosis and adenoviral infections (Huchzermeyer 2003).

Dermatophilosis (“Brown Spot Diseases”)

“Brown spot disease” has been attributed to *Dermatophilus* sp., with most of the cultures resembling *Dermatophilus congolensis*. It has been reported in both crocodiles and alligators (Newton 1992, Bounds and Normand 1991, Buenviaje et al. 1997, Buenviaje et al. 1998, Barnett and Cardeilhac 1998). Affected animals present with brown to red lesions on the skin that are usually located at the junction of the ventral abdominal scales. Over time, these lesions may become ulcerated. This disease does not respond well to antibiotic therapy, but can be prevented by practicing intensive hygiene methods.

Table 4. Bacteria isolated from *A. mississippiensis* with and without clinical signs of disease.

| Isolate | Tissue | Clinical Signs/Lesions | Reference |
|--|--|------------------------|---|
| <i>Aeromonas hydrophila</i> | Blood | Yes | Brown et al. 2001 |
| | Lungs, heart, liver, kidneys, intestines, oral cavity | Yes, No | Gorden et al. 1979 |
| | Lungs, blood | Yes | Shotts et al. 1972 |
| | Eye | Yes | Millichamp et al. 1983, Jacobson et al. 1984 |
| | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Aeromonas sp.</i> | Lungs | Yes | Clippinger et al. 2000 |
| <i>Acitenobacter calcoaceticus</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Aerobacter radiobacter</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacillus sp.</i> | Not specified | Yes | Barnett and Cardeilhac 1995 |
| <i>Bacteroides asaccharolyticus</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacteroides bivius</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Bacteroides loescheii/denticola</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Bacteroides oralis</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacteroides sordellii</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacteroides thetaiotamicron</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacteroides vulgatus</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Bacteroides sp.</i> | Water | No | Flandry et al. 1989 |
| <i>Citrobacter freundii</i> | Blood | Yes | Novak and Seigel 1986 |
| | Oral cavity | No | Flandry et al. 1989 |
| <i>Clostridium bifermentans</i> | Oral cavity, water | No | Flandry et al. 1989 |
| | Lungs | Yes | Clippinger et al. 2000 |
| <i>Clostridium clostriidoforme</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Clostridium innoculum</i> | Water | No | Flandry et al. 1989 |
| <i>Clostridium limosum</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Clostridium sordellii</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Clostridium sporogenes</i> | Blood | Yes | Clippinger et al. 2000 |
| <i>Clostridium tetani</i> | Oral cavity | No | Flandry et al. 1989 |

(Table 4. Continued)

| | | | |
|---------------------------------|-----------------------------|-----|--------------------------------------|
| <i>Clostridium sp.</i> | Blood | Yes | Clippinger et al. 2000 |
| <i>Corynebacterium sp.</i> | Tail abscess | Yes | Shotts et al. 1972 |
| <i>Dermatophilus sp.</i> | Skin | Yes | Newton 1992, Bounds and Normand 1991 |
| <i>Diphtheroid sp.</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Edwardsiella tarda</i> | Kidney, feces | Yes | Wallace et al. 1966 |
| | Fat body, pericardial fluid | Yes | Clippinger et al. 2000 |
| <i>Enterobacter agglomerans</i> | Blood | Yes | Novak and Seigel 1986 |
| <i>Enterobacter cloacae</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Enterobacillus sp.</i> | Lungs | Yes | Shotts et al. 1972 |
| <i>Escherichia coli</i> | Systemic | Yes | Russell and Herman 1970 |
| <i>Fusobacterium nucleatum</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Fusobacterium varium</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Klebsiella oxytoca</i> | Skin | Yes | Novak and Seigel 1986 |
| | Oral cavity | No | Flandry et al. 1989 |
| <i>Klebsiella sp.</i> | Lungs | Yes | Nevarez 2002-03 |
| <i>Micrococcus kristinae</i> | Blood | Yes | Brown et al. 2001 |
| <i>Moraxella sp.</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Morganella morganii</i> | Blood | Yes | Novak and Seigel 1986 |
| | Oral cavity | No | Flandry et al. 1989 |
| | Lung | Yes | Clippinger et al. 2000 |
| <i>Mycoplasma alligatoris</i> | Multiple tissues | Yes | Brown et al. 2001 , 39 |
| <i>Pasteurella haemolytica</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Pasteurella multocida</i> | Lungs | Yes | Mainster et al. 1972 |
| <i>Pasteurella sp.</i> | Oral cavity, water | No | Flandry et al. 1989 |
| <i>Peptococcus magnus</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Peptococcus prevotii</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Proteus mirabilis</i> | Blood | Yes | Brown et al. 2001 |
| <i>Proteus vulgaris</i> | Oral cavity | No | Flandry et al. 1989 |
| | Oviduct | Yes | Wallace et al. 1966 |
| | Blood | Yes | Brown et al. 2001 |
| | Lung | Yes | Clippinger et al. 2000 |

(Table 4. Continued)

| | | | |
|---|----------------------------|-----|------------------------|
| <i>Proteus sp.</i> | Blood | Yes | Novak and Seigel 1986 |
| <i>Providencia rettgeri</i> | Lung, brain, liver, spleen | Yes | Camus and Hawke 2002 |
| <i>Pseudomonas cepacia</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Pseudomonas diminuta</i> | Water | No | Flandry et al. 1989 |
| <i>Pseudomonas fluorescens</i> | Water | No | Flandry et al. 1989 |
| <i>Pseudomonas pickettii</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Pseudomonas vesicularis</i> | Water | No | Flandry et al. 1989 |
| <i>Pseudomonas sp.</i> | Lungs, Pharynx | Yes | Shotts et al. 1972 |
| | Water | No | Flandry et al. 1989 |
| <i>Salmonella typhimurium</i> | Gastrointestinal tract | Yes | Shotts et al. 1972 |
| <i>Salmonella braenderup, anatum,</i> | Cloaca | No | Shotts et al. 1972 |
| <i>Arizona spp.</i> | | | |
| <i>Serratia marcescens</i> | Skin | Yes | Novak and Seigel 1986 |
| <i>Serratia odorifera</i> | Oral cavity | No | Flandry et al. 1989 |
| <i>Staphylococcus aureus</i> | Lungs | Yes | Mainster et al. 1972 |
| <i>Staphylococcus cohnii</i> | Blood | Yes | Brown et al. 2001 |
| <i>Streptococcus sp. β-hemolytic</i> | Lungs | Yes | Clippinger et al. 2000 |
| <i>Vibrio parahaemolyticus</i> | Blood | Yes | Brown et al. 2001 |

Mycoplasmosis

Mycoplasma alligatoris is a recognized respiratory pathogen of crocodylians. It has been documented in American alligators and the broad-nosed caiman (*Caiman latirostris*) (Brown et al. 2001, Brown et al. 2001a, Pye et al. 2001). Other crocodylian species closely related to alligators may also be susceptible. Clinical signs are non-specific and include lethargy, weakness, anorexia, white ocular discharge, paresis, and edema (facial, periocular, cervical, limbs) (Clippinger et al. 2000). Necropsy often reveals evidence of pneumonia, pericarditis, and polyarthritis. Helmick et al. reported antimicrobial susceptibility for *M. alligatoris* with doxycycline, oxytetracycline, enrofloxacin, sarafloxacin, tilmicosin, and tylosin (Helmick et al. 2002). A second mycoplasma species, *M. crocodyli*, was also described in Nile crocodiles and

the lesions were similar to those observed with *M. alligatoris* (Mohan et al. 1995). Some studies have examined the use of an autogenous vaccine for *M. crocodyli*, but its efficacy is yet to be determined (Mohan et al. 1997, Mohan et al. 2001).

Mycobacteriosis

Cases of pulmonary and enteric mycobacterial infections are mentioned by Youngprapakorn et al. and Huchzermeyer (Huchzermeyer 2003, Youngprapakorn et al. 1995). These reports include *M. marinum* and *M. fortuitum* from different spectacled caimans (*C. crocodilus*), *M. avium* from an unspecified species of crocodile and from Nile crocodiles, and *M. ulcerans* from Johnston's crocodiles (*C. johnstoni*) (Huchzermeyer 2003). Many other reported cases are based on gross findings and histopathology with acid-fast positive organisms identified microscopically. The author has observed some clinical cases of suspected, yet unconfirmed, pulmonary mycobacteriosis based on necropsy and histopathologic evaluation (Nevarez 2002-03). Difficulties of growing *Mycobacterium sp.* make its definitive diagnosis a challenge. Other diagnostic tools such as PCR may also be beneficial in identification of the bacteria.

Viral Diseases

The identification and diagnosis of viruses in reptiles has been slow as compared to other species. This is in great part due to difficulties in developing diagnostic tests, as well as a lack of knowledge about what viruses affect reptiles and under what conditions. Many clinical presentations that go undiagnosed could be attributed to viruses. These may be new viruses or just unknown to occur in a particular species. In crocodylians there are only a handful of recognized viruses that have been documented over the years. Poxvirus and West Nile virus are recognized pathogens in crocodylians. Jacobson et al. described an adenovirus-like infection in captive Nile crocodiles characterized by nonspecific clinical signs, lethargy and anorexia. Conjunctivitis and blepharitis was also observed in one of two crocodiles (Huchzermeyer 2003,

Jacobson et al. 1984). Intranuclear inclusions are usually found in the liver but may also occur in the intestines, pancreas, and lung. Both horizontal and vertical transmissions have been postulated (Huchzermeyer 2003). Diagnosis is obtained postmortem and no treatment regimes have been established.

Coronavirus, influenza C virus, and paramyxovirus have been identified by transmission electron microscope in the feces of crocodilians (Huchzermeyer 2003). Herpes virus-like particles were identified by electron microscopy in the skin of a saltwater crocodile (*C. porosus*) (McCowan et al. 2004). There has been a second report of herpes virus identified from the cloaca of an American alligator via PCR (Johnson 2005). The clinical significance of these findings is unknown. Seroconversion to paramyxovirus and eastern equine encephalitis virus has also been reported (Huchzermeyer 2003).

Pox Virus

Parapoxvirus or pox-like viruses have been identified in five different crocodilian species: spectacled caiman (*Caiman crocodilus fuscus*) (Jacobson et al. 1979, Penrith et al. 1991) Brazilian caiman (*Caiman crocodilus acre*) (Ramos et al. 2002), Nile crocodile (Horner 1990, Pandey et al. 1990), saltwater crocodile (Buenviaje et al. 1992), and freshwater crocodile (*Crocodylus johnstoni*) (Buenviaje et al. 1992). Pox lesions in caimans will present as 1-3 mm diameter, gray to white, coalescing to macular. Their location on the body includes the head, palpebra, maxilla, mandible, limbs, palate, tongue, and gingiva (Jacobson et al. 1979, Penrith et al. 1991, Ramos et al. 2002). Palpebral and generalized edema can also be present. Resolution of clinical signs has been observed with and without changes in husbandry (Penrith et al. 1991, Ramos et al. 2002). In crocodiles the lesions are described as 2-8 mm in diameter, yellow to brown, wart-like, sometimes firm, and unraised to raised nodules with occasional shallow ulcers. Their location on the body includes the head, palpebra, nostrils, sides of the mouth, oral cavity,

limbs, ventral neck and coelom, and at the root of the tail (Horner 1990, Pandey et al. 1990). Resolution of lesions was reported to occur as early as three to four weeks (Horner 1990). Histopathologic findings include epithelial hyperplasia, acanthosis, hyperkeratosis, and necrosis. Borrel and Bollinger's bodies may also be visible in some cases (Jacobson et al. 1979, Penrith et al. 1991, Ramos et al. 2002, Horner 1990, Pandey et al. 1990). Secondary bacterial and fungal infections may occur concurrently. At this time there are no specific treatment recommendations. The use of an autogenous vaccine to treat poxvirus in Nile crocodiles has been reported with some success (Horner 1990). Mosquito control and following good hygiene practices are essential in preventing and controlling poxvirus outbreaks.

West Nile Virus

West Nile virus (WNV) has been reported from various crocodylian species, including the American alligator (Miller et al. 2003), the Nile crocodile (Steinman et al. 2003), and the Morelet's crocodile (*Crocodylus moreletii*) (Rubio 2003). Crocodylians likely become infected via a mosquito bite, as occurs in birds and mammals. There is also the possibility of infection after ingestion of an animal with a high viral load of WNV as demonstrated by Klenk et al. (Klenk et al. 2004). This last scenario is more likely to occur when housed outdoors and not in enclosed buildings as in most alligator ranching operations. It has been demonstrated that alligators can serve as amplifiers of WNV (Klenk et al. 2004). Although there is a lot to be learned about WNV in crocodylians, we believe that once infected they can develop high viremias and shed the virus in the feces. Fecal shedding leads to horizontal transmission of the virus. The author has observed clinical evidence for this in commercial operations. Fecal shedding and high viremias also raise the concern of zoonosis, especially in commercial operations where animals are being slaughtered and people come in contact with blood and tissues. A strict building quarantine and hygiene strategies should be implemented to prevent

spread to other animals in the facility as well as to the personnel. In the state of Louisiana, alligator producers were provided with biosecurity recommendations to help them deal with episodes of WNV and prevent spread of the disease.

Affected animals in captive operations have ranged in age from one month to over twelve months old. In younger animals, infection is usually severe and acute with as much as 60% mortality. The pattern of deaths is peracute, usually seen as a sudden onset of mortalities followed by a peak and subsequent decline in the number of deaths. However, sporadic mortalities may also be seen, especially in older animals. Clinical signs of WNV in alligators include swimming in circles, head tilt, muscle tremors, weakness, lethargy, and anorexia. Bloating and difficulties swimming have also been observed (Nevarez et al. 2005b).

Gross findings are nonspecific. Light microscopy of tissues from infected animals may reveal diffuse severe heterophilic, histiocytic, and necrotizing enterocolitis, heterophilic meningoencephalitis, necrotizing and heterophilic hepatitis, heterophilic and histiocytic splenitis, generalized heterophilic and histiocytic lymphoid folliculitis, and necrotizing and heterophilic pancreatitis. You may also observe a mild multifocal heterophilic and lymphohistiocytic interstitial nephritis, gastritis, and mild pulmonary congestion and edema. Strong immunopositivity has been observed in the brain, liver, spleen, pancreas, kidney, and gastrointestinal tract after immunohistochemistry testing for WNV. Proliferative enteritis has been diagnosed in a group of alligators positive for WNV. These animals had colon lesions tested positive for WNV via Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), culture and immunohistochemistry (Nevarez 2002-03, Nevarez et al. 2005b). The affected alligators were bloated and unable to submerge under water. In the author's opinion, this was due to a blockage caused by the fibrinous membrane in the colon leading to bloating. This same presentation has not been observed in any other cases of WNV.

There are no proposed treatments for WNV in crocodylians. Mosquito control, strict quarantine and biosecurity are essential for the prevention of WNV. Once WNV is present in any operation, it is critical to maintain affected animals and any in contact with them in strict isolation. These must not be moved to other areas or buildings where WNV has not been observed. Failure to isolate them may result in the spread of the virus. In addition, there should be strict biosecurity measures, especially in the affected areas or buildings. Tools, such as nets, feeding utensils, and boots, should be left in the affected area and not introduced to other areas or buildings. Once the infection runs its course, the surviving animals will continue to thrive; however, the duration of shedding in these cases is unknown. The author has observed antibody titers of 1:640 and 1:320 in two alligators 14 months after exposure to WNV. These results were obtained via a Plaque Reduction Neutralization test (PRNT). Definitive diagnosis of WNV infection should be based on history, clinical signs and the results of diagnostic tests. Postmortem diagnosis can be performed via RT-PCR and/or viral culture of brain, spinal cord, or liver. Immunohistochemistry also provides supportive evidence of WNV infection. This last technique may prove useful as an ante-mortem test when applied to biopsies of the colon mucosa.

Fungal Diseases

Fungal infections also occur with some frequency in captive crocodylians. Most fungi are opportunistic invaders of the integument, respiratory system, and gastrointestinal tract. Poor water quality, trauma, stress, and extreme temperatures can contribute to the occurrence of fungal disease. It is thought that most fungal infections in crocodylians are of enteric origin and occur in other tissues secondary to a stressor (Huchzermeyer 2003). It is also important to remember that fungi occur as ubiquitous organisms in nature. Therefore, it is not uncommon to isolate fungi from tissues that are in contact with water and soil, such as the skin and intestinal

tract. The presence of these fungi will vary with geographic location and management techniques that provide different environments for their growth. There are a number of fungi that have been isolated from crocodilians (Huchzermeyer 2003, Huchzermeyer et al. 2000, Buenviaje et al. 1998, Nevarez 2003, Buenviaje et al. 1994, Fromtling et al. 1979, Trevino 1972, Frelier 1985, Silberman et al. 1985, Thomas et al. 2002, Maslen et al. 1988) (Table 5). Some of these were associated with disease, while others were incidental findings. Diagnosis of fungal disease requires the identification of a fungal organism via culture or special stains of the affected tissues with concomitant histopathologic identification. Positive identification to genus and species may be difficult. Treatment is expensive and may only be affordable in a small number of animals. It also requires a long administration periods of at least two or three months. For these reasons, many fungal infections go untreated and recommendations are made for prevention of disease.

Parasitic Diseases

A number of parasites are known to affect crocodilians. Protozoa, nematodes, trematodes, and pentastomes have all been reported in crocodilians. Cestode larvae have been found in some species but no adult tapeworms are reported from crocodilians. External parasites such as leeches, flies, mosquitoes, ticks and mites can also affect crocodilians (Huchzermeyer 2003). The presence of scales has created a misconception about the possibilities for external parasitism in crocodilians; however there are a number of areas in a crocodilian's body that have soft skin and allow an external parasite to attach and/or obtain a meal. The recent cases of WNV in the American alligator have reminded us that arthropods can play an important role in disease transmission. Internal parasites are not a common problem in ranching operations where animals are maintained in concrete pools without organic substrate. Endoparasites can be a concern in wild crocodilians or those kept outdoors on organic substrates. Ectoparasites can be a problem in

both captive and wild populations. If a parasitism problem is encountered, one must assess the environment of the animals as well as the life cycle of the parasite in order to determine the best prevention and control methods.

Table 5. Fungi isolated from crocodylians around the world. This table presents isolates from multiple tissues regardless of whether a disease process was attributed to the fungus.

| Fungus | Sample/Tissue | Species | Reference |
|-------------------------------------|---------------------|-----------------------------------|---|
| <i>Acremonium</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Alternaria alternata</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Arthriniium</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Aspergillus clavatus</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Aspergillus flavus</i> | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Aspergillus fumigatus</i> | skin | Caimans (species not specified) | Huchzermeyer 2003 |
| | lungs | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| <i>Aspergillus niger</i> | not specified | <i>Crocodylus porosus</i> | Buenviaje et al. 1994 |
| | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Aspergillus ustus</i> | lungs | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| <i>Aspergillus versicolor</i> | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| <i>Beauveria bassiana</i> | lungs | <i>Alligator mississippiensis</i> | Fromtling et al. 1979 |
| | lungs | <i>Crocodylus niloticus</i> | Huchzermeyer 2003 |
| <i>Beauveria</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Candida albicans</i> | oral cavity | Caimans (species not specified) | Huchzermeyer 2003 |
| <i>Candida guilliermondii</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Candida krusei</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Candida parasitosis</i> | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| <i>Candida</i> sp. | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| <i>Cephalosporium</i> sp. | muscle | <i>Caiman crocodilus</i> | Huchzermeyer 2003 |
| | lungs | <i>Caiman sclerops</i> | Trevino 1972 |
| <i>Chaetomium globosum</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Chaetomium</i> sp | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Chryso sporium</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Cladosporium cladosporioides</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Cladosporium oxysporum</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Cladosporium</i> sp. | skin | Caimans (species not specified) | Huchzermeyer 2003 |
| <i>Cryptococcus lipolytica</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Cryptococcus luteolus</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Curvularia lunata</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Curvularia lunata varaeria</i> | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1994 |
| <i>Curvularia</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Epicoccum purpurascens</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Eurotium chevalieri</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Fusarium moniliforme</i> | lungs | <i>Alligator mississippiensis</i> | Frelier et al. 1985 |
| <i>Fusarium solani</i> | internal organs | <i>Crocodylus porosus</i> | Huchzermeyer 2003, Buenviaje et al. 1994 |

(Table 5. Continued)

| | | | |
|----------------------------------|---------------------|---|--------------------------|
| <i>Fusarium</i> sp. | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| | gingiva | <i>Caiman crocodilus fuscus</i> | Huchzermeyer 2003 |
| | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Geotrichum candidum</i> | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Geotrichum</i> sp. | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| <i>Metarhizium anisopliae</i> | lungs | Crocodile (species not specified) | Huchzermeyer 2003 |
| | lungs | <i>Osteolaemus tetraspis</i> | Huchzermeyer 2003 |
| | lungs | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| <i>Monascus ruber</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Mucor circinelloides</i> | skin | Caimans (species not specified) | Huchzermeyer 2003 |
| | gastric ulcers | <i>Osteolaemus tetraspis</i> | Huchzermeyer 2003 |
| | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Mucor</i> sp. | lungs | Crocodile (species not specified) | Silberman et al. 1977 |
| <i>Nannizziopsis vriesii</i> | skin | <i>Crocodylus porosus</i> | Thomas et al. 2002 |
| <i>Paecilomyces lilacinus</i> | liver | <i>Crocodylus porosus</i> | Maslen et al. 1988 |
| <i>Paecilomyces farinosus</i> | lungs | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| <i>Paecilomyces variotii</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Paecilomyces</i> sp. | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Penicillium citrinum</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Penicillium lilacinum</i> | lungs | Alligators and Crocodiles (species not specified) | Huchzermeyer 2003 |
| <i>Penicillium oxalicum</i> | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1994 |
| <i>Penicillium</i> sp. | not specified | <i>Crocodylus porosus</i> | Huchzermeyer 2003 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Phoma</i> sp. | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Syncephalastrum racemosum</i> | skin | <i>Alligator mississippiensis</i> | Nevarez 2003 |
| <i>Syncephalastrum</i> sp. | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| <i>Trichoderma</i> sp. | skin | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Trichophyton</i> sp. | skin | <i>Alligator mississippiensis</i> | Huchzermeyer 2003 |
| <i>Trichosporon beigelii</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Trichosporon capitatum</i> | intestinal contents | <i>Osteolaemus tetraspis</i> | Huchzermeyer et al. 2000 |
| <i>Trichosporon cutaneum</i> | skin | <i>Crocodylus porosus</i> | Buenviaje et al. 1998 |
| <i>Trichosporon</i> sp. | tongue, gingivae | <i>Crocodylus niloticus</i> , <i>C. fuscus</i> | Huchzermeyer 2003 |

Nutritional Disease

Nutritional diseases are probably not observed as frequently now as they were in the past. Increased understanding of the biology, natural history, and dietary requirements of the different crocodilian species has allowed better feeding schemes. Over time there has been a push to develop commercial diets that will allow for high feed to weight gain ratios. These are available for feeding American alligators, and although not perfect, do allow producers to grow captive hatched alligators up to 36 inches in length in 12 months or less. In zoological institutions, the goal is not rapid growth but rather steady and healthy development. In these cases, there is

common use of fresh/frozen prey (e.g., poultry, swine, beef, fish, nutria, native prey) with bones as a source of calcium. This is also a common practice in crocodile farms. It is essential to use a reputable source and ensure that no lead shots have been used to kill the prey item in order to avoid Lead toxicity. Despite better feeding schemes, there are still some presentations that can be associated with nutritional deficiencies.

A common nutritional disease of reptiles is metabolic bone disease (MBD). There are various manifestations and pathogenesis for MBD but it is usually associated with a calcium/phosphorus imbalance that leads to increased bone resorption. Calcium and vitamin D₃ deficiencies are the two main etiologies for MBD. Vitamin D₃ deficiencies may be associated with a lack of exposure to UVB light. There are various light products in the market that can be used to provide reptiles with UVB lighting. Clinical signs of MBD include weakness, lethargy, kyphosis, scoliosis, osteodystrophy, pathologic fractures, paresis, and tooth decalcification. The development of MBD is most often reported in herbivorous or omnivorous reptiles. MBD is not common in commercial crocodilian operations, even in those where animals are raised in the dark with no source of UVB. One explanation for this may come from the carnivorous nature of crocodilians, which may allow them to obtain vitamin D₃ from the diet without a true requirement for UVB. However, if an appropriate calcium source is not offered, these animals will likely develop MBD. The author has observed adult alligators grown in enclosed buildings with no light source and a commercial diet with some meat supplementation to develop normally. However, anecdotal comments from various ranchers indicate that the animals appear to thrive better if exposed to sunlight. To date, metabolic bone disease continues to be a rare occurrence in alligator ranches. Nonetheless, this is an area where more research is needed to determine the UVB and calcium requirements of crocodilians.

Gout has been reported in crocodylians. It can occur as either an articular or visceral form. Some predisposing factors for gout include high protein diets, dehydration, and stress. Limb paresis/paralysis and joint enlargement may be observed with chronic gout; otherwise the clinical signs are non-specific. Ariel et al. reported a case of gout with concurrent hypovitaminosis A in crocodile hatchlings (Ariel et al. 1997).

Deficiencies of vitamins A, B, C, or E can also lead to a variety of musculoskeletal disorders. These are thought to be less common when commercial diets are fed in addition to meat products.

Respiratory Disease

Respiratory disease is one of the most common presentations of captive crocodylians in commercial operations, second only to integumentary disease. Gross pathologic lesions in the lungs are a common finding during necropsies. While many of these findings are incidental, some will show clinical signs before death. Clinical signs in affected animals can be non-specific anorexia, lethargy, and weakness, or be more system related and include dyspnea, tachypnea, nasal secretion, excessive basking, respiratory stridor, and abnormal swimming (either in circles or on one side of the body). In some cases, the animals may appear neurologic as they become weak and ataxic. A clear distinction must be established between respiratory disease and neurological disease. On the other hand, respiratory disease may be secondary to neurologic disease as a consequence to the weakness that leads to aspiration. Most respiratory infections are either bacterial or fungal in origin. Upper respiratory diseases, including rhinitis and pharyngitis, have also been reported in crocodylians (Huchzermeyer 2003).

Neurologic Disease

Neurologic diseases are not common in crocodylians; however, the etiologies implicated with this presentation deserve special attention. The most common neurologic presentation will

relate to abnormal swimming behavior (e.g., swimming in circles, swimming on one side of the body). Once outside the water, these animals may show signs of lethargy, ataxia, head tilt, and muscle tremors. Anorexia will also be observed and is perhaps an early sign observed by personnel working with the animals. Any infectious agent affecting the nervous system can lead to this presentation. The most recent etiology of neurologic signs in crocodylian species is WNV. This should be included as a rule out for any crocodylian that presents with neurologic signs and is housed in an area with prior history of WNV. Hypoglycemia has also been reported as a cause of neurologic signs in alligators (Wallach et al. 1967). Another cause of neurologic signs may be associated with increased ammonia/oxygen depletion in enclosed buildings, especially during the summer. The author has observed neurologic signs and deaths of American alligators from a commercial operation where a building was not cleaned on schedule. The water in the building had not been changed in 72 hours and there was evidence of food left over from previous feedings. The owner found both dead alligators and some with neurologic signs. Necropsy and histopathologic evaluation pointed towards toxicity as the cause of death. Both dead and live animals tested negative for WNV. All other buildings were cleaned on schedule and had no evidence of disease. Mortalities decreased after cleaning of the affected building. Based on the history, clinical observations, and histopathologic findings, we believe these animals showed neurologic signs as a result of either increased ammonia level in the water, oxygen depletion in the air, or both.

Musculoskeletal Disease

Musculoskeletal disease can occur due to changes in the incubation temperature or humidity, trauma from fighting, transport, or restraint and from infectious diseases (Figure 13). Deformities of the head, limbs, and tails are common malformations influenced by incubation rather than true genetics (Figure 14). Fractures and limb amputations can occur due to trauma

and fighting. Many of these heal without major complications. This can be seen in captive as well as wild crocodilians. In severe cases, trauma can lead to nerve or muscle damage and paresis or paralysis. The most common infectious disease known to have effects on the musculoskeletal system is mycoplasmosis. Both *M. alligatoris* and *M. crocodyli* can cause polyarthritis in affected animals.

Gastrointestinal Disease

Ingestion of foreign bodies, gastric ulcers, enteritis, and trauma to the oral cavity are some of the most common gastrointestinal diseases reported in crocodilians. Anorexia usually accompanies gastrointestinal disease. Ingestion of foreign bodies occurs in both wild and captive crocodilians. Construction, malfunction of water pumps and filters, forgotten objects in the enclosure, and tossed objects by the public are some of the sources of foreign bodies in captive scenarios. Sharp objects, such as nails, can cause severe problems, while other objects may pass



Figure 13. Mandibular trauma in a wild alligator. This type of trauma is usually observed in males during the breeding season.



Figure 14. Maxillary deviation in a captive hatched alligator. These abnormalities are believed to be caused by irregularities in temperature and or humidity during the incubation period.

without difficulties. Infectious enteritis is usually diagnosed post- mortem based on gross and histopathologic observations. Crocodilians appear to have an aggressive response to insult of the gastrointestinal tract. Grossly, you may observe accumulation of fibrinous or fibrous material and/or necrosis of the mucosa. (Figure 15) In some instances, this can even lead to obstruction due to accumulation of fibrinous/fibrous material. Fecal impactions and torsions are probably rare but can also occur in crocodilians. Gastric ulcerations may be associated with stress and diet. The intestinal tract of crocodilians appears to contain a significant amount of gut associated lymphoid tissue similar to Peyer's patches. In the author's opinion, there is opportunity for an aggressive inflammatory response to infectious agents in the intestinal tract.

Integumentary Disease

Integumentary disease is likely the most important disease process affecting captive crocodilians. Water quality, temperature, and stressors contribute to the occurrence of integumentary diseases. The economic impact in commercial operations can be devastating. Secondary bacterial and fungal dermatitis are a major problem in these operations. Lacerations,

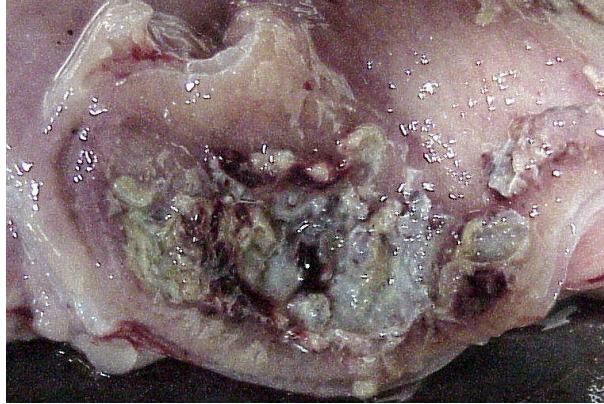


Figure 15. Fibrin deposition and necrosis of intestinal mucosa in an alligator.

abscesses, and draining tracts can serve as a nidus for microorganisms, especially fungi and bacteria. An additional factor in captive operations is the accumulation of fat in the upper water column. This fat creates a slime layer on the walls and water surface, which is then transferred to the skin of the animals creating an ideal environment for fungal and bacterial growth. This is more of a problem when meat is the sole source of food or is provided in addition to a commercial diet. A surfactant disinfectant can be used to break down the fat accumulation in the water as well as on the walls of the enclosure. In most instances, both bacteria and fungi will be present in these skin lesions. It is important to recognize the problem early on to institute appropriate cleaning measures and possible therapy. Culture and sensitivity testing can be frustrating in these cases due to the mixed flora found in the lesions. Antimicrobial therapy is indicated if you suspect bacterial involvement. The use of systemic antifungal medications is cost prohibitive in commercial operations, but may be used if only a small number of animals are affected. Improved hygiene, water quality, stable temperatures, and decreased stress are essential for managing these cases. In most instances those animals that are already affected may not recover but you can prevent the occurrence of disease in new animals by improving the husbandry methods.

Various alligator ranches in Louisiana have reported hatching animals with normal pigmentation that start becoming white after a few weeks of life (Figure 16). Physical examination of these animals does not reveal any abnormalities other than an apparent depigmentation/hypopigmentation of the skin. The white discoloration is not like that seen in fungal or bacterial dermatitis. Upon palpation of the skin, it appears thinner and has a flaky nature similar to that observed in leucistic alligators. Affected animals do not appear to grow at different rates than normally pigmented animals. The processing of the hides is not affected by the pigment deficit. A nutritional deficiency or genetics are suspected in this presentation. There are anecdotal reports of improvement of the condition after vitamin supplementation.

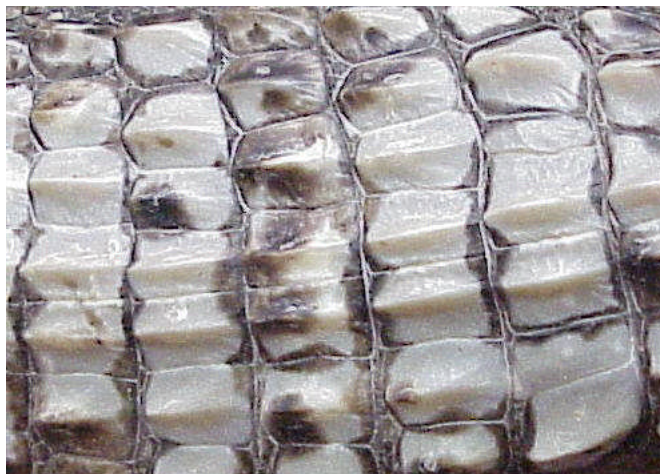


Figure 16. Depigmentation/hypopigmentation observed in a captive hatched alligator.

Toxicities

Toxicities are not common in captive crocodylians. However, there have been cases of lead toxicity in alligators being fed lead-shot nutria (Camus et al. 1998). Clinical signs included weakness, lethargy, anorexia, and death. Alligator ranchers/farmers are now more diligent in inquiring about the source and kill method of nutria before feeding the animals. Wild alligators on the other hand can be exposed to a number of toxic substances, mostly pesticides and chemicals. Many of these are thought to be endocrine disruptors that affect the development and

function of the reproductive organs. This in turn can lead to developmental defects in young crocodilians (Vonier et al. 1996, Lind et al. 2004, Stoker et al. 2003, Guillette et al. 2002, Rauschenberger et al. 2004). There is an abundance of information to support the impact of environmental contamination on crocodilians as well as other species. This reiterates the critical state of the natural habitat of many species around the world. Crocodilians have served as sentinels for anthropogenic changes that ultimately may come back to affect human health.

Runting

Runting is a phenomenon observed in captive crocodilian operations. It describes a general state of unthriftiness, lack of growth and weakness. There is a visible size difference in same age animals between the runts and the otherwise healthy ones. It is not unusual to find some buildings in commercial operations that contain the runt animals for that year. It is also in this group that disease problems may be observed with higher frequency. Dominance by other animals, environment, and even incubation factors contribute to the presence of runts (Allstead and Lang 1995).

West Nile virus History

West Nile virus is a Flavivirus found within the Japanese encephalitides virus group, which also includes Cacipacore, Koutango, Alfuy, Kunjin, Japanese encephalitis, Murray Valley encephalitis, St. Louis encephalitis, Usutu, and Yaounde viruses (Mackenzie et al. 2002). Most flaviviruses are positive sense single stranded RNA viruses that measure between 40 and 60nm in diameter and have an enveloped icosahedral nucleocapsid (CDC 2003). WNV virus was first identified in 1937 during an epidemiologic study of yellow fever in Africa (Smithburn et al. 1940). During this investigation, serum from affected individuals was inoculated intracerebrally into mice and a subinoculation then performed from mice that died after the original inoculation (Smithburn et al. 1940). Smithburn et al. (1940) reported the isolation of various transmissible

agents, one of which they named WNV. This newly identified virus, WNV, was isolated from a woman in the West Nile province of Uganda who was suffering from a mild case of febrile illness (Smithburn et al. 1940). At the time, the febrile illness was thought to be associated with yellow fever, and no additional history to relate the clinical signs to the newly discovered virus was obtained (Smithburn et al. 1940). Since this discovery of WNV, sporadic cases were reported around the Middle East, Africa, Europe, and Asia; however, it wasn't until 1951 that the first documented epidemic of WNV was reported in Israel (Mackenzie et al. 2002). This led to continued interest in the virus and various research projects to determine its origins, cycle, and pathogenicity. From this original research, it was determined that the epidemiology of WNV included mosquitoes and birds, and that human and horses were incidental hosts (Mackenzie et al. 2002). A serological survey at the time demonstrated greater than 60% exposure in humans along the Egyptian Nile (Mackenzie et al. 2002). By 1950, *Aedes albopictus*, *Culex pipiens*, and *Culex tritaeniorhynchus*, three species of mosquitoes, had been shown to experimentally transmit WNV (Philip and Smadel 1943, Kitaoka 1950). Additional mosquito species from which WNV was isolated during the 1950s included *Culex antennatus* and *Culex univittatus*, which some considered to be the primary vector in many areas of Egypt (Hayes 2001). During the 1960s, *Culex modestus* and *Culex vishnui* were implicated as vectors of WNV in France and India, respectively (Hayes 2001). Today *C. pipiens pipiens* is considered to be the most important mosquito vector in North America and parts of Europe (Hayes 2001). As of 2005, WNV had been isolated from over 60 different species of mosquitoes (CDC 2005).

The pathogenicity of WNV in humans was first described in 1954 after experimental inoculation of humans with the Egypt 101, 21, and 19 WNV isolates. The original plan of the authors was to evaluate the possible antineoplastic properties of the virus (Southam and Moore 1954). The clinical signs reported in the human patients during the study ranged from being

asymptomatic to fever and encephalitis (Southam and Moore 1954). This early study of WNV infection in humans led to the conclusion that humans were likely incidental hosts and did not play a significant role in the cycle of WNV (Southam and Moore 1954). Southam and Moore also showed that viremia in humans could be detected from 24 hours to 12 days after inoculation with WNV, which is consistent with the 3 to 15 day incubation period that is generally accepted today. In the original experimental studies, the authors were also able to establish a correlation between the persistence of a viremia and the severity of clinical disease (Southam and Moore 1954, Mackenzie et al. 2002). The histopathologic descriptions of WNV infections in deceased patients were not very clear, and were confounded by the neoplastic disease that the patients initially had before being infected with WNV.

The epidemiology of WNV was also of great interest at the time. It was believed that the increasing density of human populations, coupled with increasing land cultivation in rural areas and increased prevalence of mosquitoes and birds, all played a role in the cycle and epidemiology of the disease (Mackenzie et al. 2002). The presence of mosquitoes, and consequently WNV in birds and humans was a link identified very early on during the study of the WNV epidemiology. Taylor et al. (1956) observed a progressive increase in WNV seropositivity with age amongst children in the Sindbis district of Egypt in 1950 (Taylor et al. 1956). It was then proposed that WNV must be endemic in areas where such findings are observed, and that the virus must over-winter in order for that endemicity to be maintained (Taylor et al. 1956). Taylor et al. (1956) also observed that while migratory birds may play a role in the epidemiology of WNV, they were unlikely to be the source of yearly outbreaks since the areas more frequented by migratory birds along Egypt's coast also had the lowest antibody rates. They further proposed three over-wintering methods for WNV: hibernating mosquitoes, other arthropods such as ticks, and small numbers of mosquitoes that remain active during the

winter (Taylor et al. 1956). In their study, they determined that there was no evidence for hibernation of the main *Culex* species in Egypt. They were also unable to isolate WNV from over 26,000 ticks from 8 different taxa (Taylor et al. 1956). They concluded that mosquitoes continued to survive during the winter in Egypt and presented evidence of *Culex* collections during winter time from which WNV was presumably identified but not confirmed. An additional piece of evidence that suggested this cycle was that the children seroconverted for WNV via complement fixation during April and May, and thus must have been exposed to WNV during the preceding winter months (Taylor et al. 1956). While some of the evidence could not be confirmed, the study presented some of the earliest evidence for the same over-wintering theories that are now being proposed in an effort to explain the endemicity of WNV in certain areas of the United States. Taylor et al. (1956) went on to present a hypothetical life cycle for WNV that said mosquitoes were the vectors for the disease, birds the primary hosts, and humans a secondary host unable to maintain the cycle without the presence of birds. Domestic quadrupeds were also mentioned as being frequently infected based on the presence of antibodies, but not reaching high enough viremias to be of significance in the propagation of the virus (Taylor et al. 1956). The Taylor et al. (1956) study concluded that in the endemic areas of the Nile Delta, WNV is “a childhood disease with yearly peaks of transmission during mid-summer” with the impression of WNV being a “self-limited, non-fatal febrile disease rarely associated with definite manifestations of encephalitis” (Taylor et al. 1956). In addition, they state that “on both experimental and ecological grounds the main cycle of WNV is probably through mosquitoes and birds in which man may become involved and domestic quadrupeds are probably tangential or dead-end infections” (Taylor et al. 1956). Finally, they reported that there is “evidence for believing that the virus over-winters through a process of retarded transmission by the mosquitoes that remain active throughout the colder months” and that “non-endemicity in

the northern Delta may be accounted for by less dense human and bird populations and the scarcity of what appears to be the most important vector, *C. univittatus*” (Taylor et al. 1956). This study was perhaps one of the most important of its time for identifying some of the same concepts that today are thought to play a role in the ecology and epidemiology of WNV.

Since the original epidemic in Israel, additional outbreaks in both animals and humans have been reported in Israel, Egypt, France, and South Africa. Continued reports of sporadic cases were also reported throughout Africa, Europe, Asia, and the Middle East well into the late 1990s and the early part of the 21st century (Smithburn et al. 1940). It wasn't until 1999 that the first cases of WNV were reported in the United States from the area of northern Queens in New York City (Nash et al. 2001, Ludwig et al. 2002, MMWR 1999a, MMWR 1999b). During August of 1999, arboviral encephalitis, characterized by meningoencephalitis with muscle weakness, was reported to the New York City Department of Health (Nash et al. 2001, MMWR 1999a). The original report of these cases led to identification of a cluster of six patients with encephalitis (MMWR 1999a). Five of the six had profound muscle weakness based on axonal neuropathy by electromyogram. Four of these patients also required respiratory support (MMWR 1999a). Initial testing revealed antibodies to St. Louis Encephalitis virus (SLE) via an IgM- capture enzyme linked immunosorbent assay (ELISA), and led to active surveillance for arboviruses as well as treatment for mosquitoes around the four square mile area from which the cases originated (MMWR 1999a). A case definition was also established: “presumptive diagnosis of viral encephalitis with or without muscle weakness or acute flaccid paralysis, Guillain-Barre syndrome, aseptic meningitis, or presence of the clinical syndrome characterizing the initial cluster of cases in a patient presenting after August 1” (MMWR 1999a). In addition, increased mortalities of birds and other wildlife were observed before and after the report of human cases (MMWR 1999a, Ludwig et al. 2002). Testing of both animal and human samples

by polymerase reaction (PCR) and consequent genomic analysis revealed the presence of a West Nile-like virus (MMWR 1999a). By October 5, 1999, the human cases increased to 50, with five deaths, and crows were being recognized as sentinels for the geographic distribution of the virus (MMWR 1999b). Mosquito collection was also initiated early on as part of the surveillance methods.

The response by the city of New York to this outbreak was characterized by an immediate active surveillance plan that included testing of mosquitoes and wild animals as well as a public outreach program. Telephone hotlines were established to address public concerns and DEET (N, N-diethyl-meta-toluamide) mosquito repellent was distributed along with educational material about how people could protect themselves against mosquito bites (MMWR 1999a). In addition, active spraying for mosquitoes was initiated in the affected areas.

Of particular interest in the history of WNV is the fact that outbreaks typically occurred in rural areas and that disease was characterized as a mild febrile illness. This changed during 1996-1999, when three major epidemics occurred in urban areas of Romania, Russia, and the United States. These outbreaks were also characterized by the presence of severe neurological disease and increased mortality in case patients, and an increased mortality in birds (Hayes 2001). In all three outbreaks, *C. pipiens* was determined to be the primary vector for WNV transmission (Hayes 2001).

Today, a lot more is known about WNV and its epidemiology in humans. As opposed to earlier studies which reported WNV as being a childhood disease with signs consisting of mild febrile illness (Taylor et al. 1956), today WNV is recognized as a disease that can have higher mortalities and morbidity amongst the adult and elderly populations in urban areas (Nash et al. 2001, Weinberger et al. 2001). Nash et al. (2001) also proposed that the more severe morbidity and mortality observed during 1999 in New York City may have been in part due to the fact that

the population of individuals in the area had naïve immune systems, and that more severe disease could be expected in non-endemic areas. These observations have changed the way that WNV is perceived around the world and led local, state, and federal agencies to work aggressively towards the prevention and control of WNV.

With an increase in morbidity and mortality has come a better understanding of the pathology of WNV in both humans and animals. Clinical signs of WNV infection can range from being absent to fever, myalgia, headache, conjunctivitis, lymphadenopathy, anorexia, vomiting, photophobia, arthralgia and skin eruptions (Anderson et al. 2004, Asnis et al. 2001, Hayes and Gubler 2006). The skin eruptions are of particular importance to our study because they reveal a dermatological component to WNV infection similar to what we have observed in alligators. Other less common clinical manifestations include hepatitis, pancreatitis, rhabdomyolysis, myocarditis, cardiac dys-rhythmias, orchitis, uveitis, vitritis, optic neuritis, and chorioretinitis (Hayes and Gubler 2006). The primary histopathologic finding associated with WNV infection in humans is encephalitis or meningoencephalitis (Asnis et al. 2001). Specific cellular changes include neuronal loss, perivascular inflammation, microglial nodules, neuronophagia, and necrosis (Hayes and Gubler 2006, Hayes et al. 2005). These changes predominate in the brainstem, deep gray nuclei, and anterior horns of the spinal cells (Hayes and Gubler 2006). Muscle necrosis and perivascular inflammation without major inflammation has also been reported in patients suffering from paralysis associated with WNV (Hayes and Gubler 2006). In humans, the histopathologic changes appear milder than infections with other flaviviruses and infections in animals (Sampson et al. 2000). The pathology of WNV in animals is quite varied. Some of the microscopic changes reported in birds infected with WNV include lymphoplasmacytic, histiocytic encephalitis and myocarditis, lymphoplasmacytic chorioiditis, heterophilic and lymphoplasmacytic thyroiditis and adrenalitis, hepatocellular necrosis, and

perivascular cuffing amongst others (Wünschmann et al. 2005). In horses the lesions are predominantly in the central nervous system (CNS), and are characterized by perivascular cuffing with lymphohistiocytic inflammation and hemorrhage of the CNS (Castillo-Olivares and Wood 2004). In alligators, WNV has systemic effects and acute lesions are primarily heterophilic in nature (Nevarez et al. 2005b).

A diagnosis of WNV infection in a human patient is mainly done by serologic testing, more specifically IgM via a WNV ELISA. This ELISA can cross-react with antibodies to other flaviviruses, so a positive results should be confirmed by a WNV plaque reduction neutralization assay (PRNT), ideally on paired sera (Hayes and Gubler 2006). In cases showing neurologic signs, CSF can also be tested using the ELISA. Polymerase chain reaction technology can also be used to test for the virus in serum or CSF. Postmortem diagnosis may be performed by RT-PCR, immunohistochemistry or viral culture coupled with histopathologic findings. Currently there is no specific treatment for WNV infection. In the presence of infection, supportive therapy must be initiated in both humans and animals. In cases of mild febrile illness, recovery will usually occur within several days while those suffering from severe illness without neurologic signs usually recover within weeks to months (Hayes and Gubler 2006). Those with neurologic signs but without focal deficits usually recover completely, but once encephalitis and flaccid paralysis ensue, the prognosis is poor (Hayes and Gubler 2006).

Prevention and control of WNV is primarily aimed at mosquito control. Active mosquito surveillance and control programs are in place throughout the United States in order to control mosquito levels and the exposure to WNV in the human and animal population. These programs also rely on the public being conscientious about decreasing mosquito breeding habitats and protecting themselves by using repellents and avoiding outdoor activities during peak hours for mosquito activity. Ultimately, WNV will remain an endemic disease in the United States, and

the morbidity and mortality associated with infection should subside if it follows the same trend observed in other countries. There are WNV vaccines approved for horses but none for humans or other animal species. These same vaccines have been studied in animal species with varying degrees of efficacy. So far there has been no vaccine developed for use in humans although this may change in the future.

An additional method of preventing the dissemination of WNV is through active surveillance to identify the virus early on before it spreads through an area. Sentinel chickens have been used as a method to monitor for WNV activity. Birds are placed in areas where WNV may appear and serially tested for the presence of seroconversion. Mosquito surveillance has become one of the most important tools in preventing and controlling WNV. This method tests pools of mosquitoes that have been trapped in areas where WNV may be suspected to occur. The tests performed on the mosquito pools detect the antigen itself and therefore serve as a more accurate tool to identify the active virus in a region as opposed to exposure. Results of mosquito surveillance for WNV are used to direct the efforts of mosquito control agencies. The information will provide these agencies with a priority of areas that should be sprayed for mosquitoes first.

Besides virus surveillance and mosquito control, an educational campaign in affected states has been an equally important part of WNV prevention. Individuals in a community must understand the risks of infection and come together to minimize conditions that promote mosquito breeding. In addition, individuals must protect themselves against infection via the use of mosquito repellents and by minimizing outdoors activities during peak mosquito hours in their area. All these aspect working together will play a key role in decreasing the morbidity and mortality of WNV during future outbreaks.

CHAPTER 1: LYMPHOHISTIOCYTIC PROLIFERATIVE SYNDROME OF ALLIGATORS: PRELIMINARY INVESTIGATION

Introduction

The Louisiana alligator industry is an important resource for the state. The industry has been used to establish a successful conservation program for the American alligator (*Alligator mississippiensis*) in Louisiana, and a financial resource for individuals in economically depressed regions of the state. Since the inception of the alligator aquaculture industry in Louisiana, producers have experienced significant fluctuations in the monetary value of alligator hides. The introduction of disease or any factor that could affect the production of high quality hides could be financially devastating.

“PIX” is a generic term used to describe a small gross lesion in alligator hides that appears as if someone has struck the skin with an ice pick (Cardeilhac 2001). These lesions reduce the overall value of the hide, and in some cases completely devalue the hide. Because of the investment placed into producing market size alligators, losses associated with this “disease” can be significant and devastating to the ranchers.

According to previous work generated by the LA Department of Wildlife and Fisheries in collaboration with Dr. Paul Cardeilhac (University of Florida College of Veterinary Medicine), the first reports of “PIX” in captive alligators occurred in Florida in 1999. These reports were limited to a single farm. Although there was concern that this “disease” might be introduced into Louisiana, there were no reports made through 2001. The first report of “PIX” in Louisiana was made in 2002. Initially, the reports were sporadic, and there were no preliminary estimates of the prevalence of “PIX” in the state. Alligators with gross “PIX” lesions were presented in early 2002 to the Louisiana State University School of Veterinary Medicine (LSU SVM) for examination. Histopathology of the gross lesions revealed a consistent lymphohistiocytic proliferative lesion. Consequently, “PIX” was re-named as lymphohistiocytic proliferative

syndrome of alligators (LPSA). Initial differentials for this syndrome included: infectious disease, immune-mediated disease, allergens, nutritional deficiencies, toxins, and neoplasia. The current study was initiated as a first attempt to characterize the epidemiology of LPSA in Louisiana alligator farms.

Materials and Methods

A cross-sectional study was performed to estimate the prevalence of LPSA in Louisiana alligator farms. The prevalence of LPSA was determined based on the presence of both gross and microscopic lesions. Alligator farms were selected based on a previous history of LPSA being described at the farm. Six farms were evaluated in this study. The farms were from geographically distinct areas within the state. The production size of the alligator farms were variable, and ranged from 5,000 – 50,000 alligators produced annually. All the farms raised their alligators indoors. Four farms used rectangular buildings and two farms used round buildings. The alligators were maintained at temperatures between 29 - 32°C (85 - 90°F). Stocking densities for the alligators varied within farms based on size and time of year and were in accordance to recommendations from the Department of Wildlife and Fisheries. All of the farms used open water systems, in which the water within the pens was removed and replaced with fresh water. The three water sources for alligator farms were city water, well water, and bayou water, or a combination of these. Water was pre-heated prior to being provided to the alligators at all farms. All of the farms fed their alligators a commercial diet, either Burris (4/6) or Lone Star (2/6). Both diets are similar in composition but vary in the size of the pellets and the length of time the pellets will remain afloat in water. At 4 farms the diet was supplemented with chicken, fish, or beef liver; however, this could vary through the year depending on prices and availability. All farms report adding bleach to the water on a subjective manner. The subjective use of salt and/or antibiotics was also reported by four farms.

A complete and thorough diagnostic evaluation was planned for 50 alligators, 25 with gross lesions (cases) and 25 without gross lesions (controls). The diagnostic tests performed for this study included: a complete blood count, plasma biochemistry analysis, protein electrophoresis, heavy metal screening, bacterial and fungal cultures of specific lesions, and histopathology. Electron microscopy and herpes virus PCR were also performed on selected tissues. An additional 32 alligators, 16 cases and 16 controls, were also sacrificed for necropsy only.

Histopathology

A complete necropsy was performed on 82 alligators, and the following tissues collected: brain, spinal cord, heart, liver, gall bladder, lung, esophagus, stomach, small intestine, pancreas, large intestine, mesentery, kidney, ureter, adrenal gland, gonad, skin, bone marrow, spleen, tonsil, conjunctiva, thyroid, eye, muscle, and nerve. All of the tissue samples were placed in 10% neutral buffered formalin. The samples were shipped to Northwest Zoo Path (654 W. Main St. Monroe, WA 98272) for processing and interpretation.

Electron Microscopy

Electron microscopy (EM) is a diagnostic tool that provides a microscopic view of tissues that is far beyond the capacity of a light microscope. Specifically, EM provides the opportunity to directly examine individual cellular components and small organisms such as viruses and bacteria that may otherwise be missed with light microscopy. The use of EM in the study would aid in the identification of possible etiologic agent(s) of LPSA. Selected tissue samples with obvious lesions (N=5) were also analyzed by Northwest Zoo Path.

Fungal Isolation

Skin samples with gross lesions consistent with LPSA were collected at necropsy and cultured for fungi at the LSU SVM. The exterior and interior surfaces of the skin sample were

sterilized by immersion in alcohol followed by flaming. Each sample was cut into smaller pieces and mixed with a maximum of 0.5ml sterile saline before plating. Saboraud-dextrose, mycocol, and potato flake agars were used to isolate potential fungi. Plates were maintained at room temperature in the dark for a minimum of 14 days. The cultures were observed daily. Isolates were sub-cultured a second time to obtain pure cultures. Samples were shipped to the Fungus Testing Laboratory at the University of Texas (7703 Floyd Curl Drive San Antonio, TX 78229-3900) for identification.

Bacterial Isolation

Bacterial cultures were performed at the LSU SVM using routine methods. The skin samples used for the fungal cultures were also used for the bacterial cultures, although no saline was added to the bacterial samples. Samples were placed on TSA with 5% Sheep blood agar and MacConkey culture media and incubated at 37°C for 24-48 hours under aerobic conditions. Bacterial isolates were characterized by colony morphology, Gram-stain characteristics, and biochemical processes.

Plasma Biochemistries

Plasma biochemistries can provide important physiologic information regarding the general health status of an animal. Blood was obtained from the supravertebral sinus of 46 alligators. Each sample was placed into a lithium heparin vacutainer tube and centrifuged on site. The plasma was removed, placed into sterile cryovials, and transported on wet ice to the LSU SVM for analysis.

Protein Electrophoresis

Protein electrophoresis is a diagnostic test used to separate total plasma protein into its various components. This test is not widely performed in reptiles, but could provide insight towards diagnosing a specific etiology or group of etiologies and provide information about the

chronicity of a disease process. We elected to perform this test after detecting total protein, albumin, and globulin alterations in preliminary plasma biochemistries performed in a pilot study. Plasma samples were shipped to the University of Miami for testing.

Complete Blood Counts

Complete blood counts (CBC) are used to evaluate the erythron and white blood cells patterns of a patient. This diagnostic test is invaluable in characterizing inflammatory responses in an animal. Complete blood counts were performed at the LSU SVM on 45 alligators using standard methods. In short, an eosinophil unopette system (Becton Dickinson and Company, Franklin lakes, NJ 07417) was used to estimate the heterophil and eosinophil counts of the animals, and a differential estimated from a Wright-Giemsa stained blood smear.

Heavy Metals and Trace Element Screening

Heavy metals can cause a severe inflammatory response when they are disseminated into tissues. The type of response elicited by heavy metals may be similar to that described in LPSA. Therefore, blood and tissue samples were collected from alligators and evaluated for the presence of various heavy metals. The blood samples were collected prior to the alligators being euthanized, while the tissue samples were collected at necropsy. The samples were processed at the Louisiana Veterinary Medical Diagnostic Laboratory.

Water Analysis

Water samples were obtained from each of the pens that housed the study subjects. Approximately 100mls of water was collected in sterile plastic containers that could be sealed tightly. The samples were then transported on wet ice to the LSU Agricultural Chemistry Laboratory for processing. The following parameters were measured: pH, ammonia, bromide, fluoride, nitrate, phosphate, sulphate, total solids, suspended solids, and dissolved solids.

Herpes Virus PCR

During sampling for this project, a subset of tissue samples was sent to the University of Florida to test for herpes and iridoviruses. These preliminary tests revealed the possible presence of a herpes virus in the tissues. Follow up work was performed at the LSU SVM. A consensus primer PCR was used based on a protocol described by van Devanter et al. (van Devanter et al., 1996). This protocol was the same one used at the University of Florida and has the advantage of being able to detect and identify herpes viruses without prior DNA information.

Statistical Methods

Descriptive statistics were generated for each parameter. 95% binomial confidence intervals (CI) were calculated for proportion data. A Shapiro-Wilk test, and the kurtosis and skewness of the data, were used to evaluate the distribution of continuous data. Data that was normally distributed was reported as a mean, while those not normally distributed as a median. A Kruskal-Wallis ANOVA was used to compare the various data between farms. A $p < 0.05$ was considered statistically significant. SPSS 11.0 (SPSS Inc., Chicago, IL) was used to analyze the data.

Results

Histopathology

The majority of the alligators collected from the farms had lesions consistent with LPSA in multiple organs (Table 6). Additionally, rhabdomyolysis and vacuolar changes in the liver and adrenal glands were observed in a number of the alligators necropsied for this study (Table 7). Based on the 95% CI, the organs that were most often affected were the intestine, lungs, and stomach. The pathologist reported that the lesions identified in these alligators were similar, and were characterized by an infiltration of lymphocytes and histiocytes. Initially, the findings from the histopathologic examination suggest that our control alligators also had lesions consistent

with LPSA; however, we know understand that lymphoid aggregates are a normal finding in the intestines, lungs, and stomach of healthy alligators. If we omit the histopathologic findings in these tissues, as they are normal findings, we can see that the 44% prevalence of skin lesions are from the group of animals with gross lesions. This provides a more clear assessment and shows that two distinct groups of animals, one with gross skin lesions and one without gross skin lesions were indeed collected.

The combination of the histopathologic findings being strongly suggestive of an infectious agent, and a report from Florida suggesting that a fungal agent may be responsible for LPSA, led us to specifically evaluate these lesions for infectious agents. There was no evidence of fungal disease in the samples. However, acid fast stains were positive in the lungs of two alligators (2.4%, 95%CI: 0-5.7%), suggesting the possible presence of *Mycobacterium* sp. This bacterium is fastidious, requiring specific culture methods that were not implemented in this study. Because these bacteria were not found consistently, it was not considered to be unrelated to LPSA. However, further study to the role of these bacteria should be performed if more consistent lesions are found. No viral disease was identified using light microscopy; however, perivascular cuffing, a common pathologic finding with infectious processes, was evident in some of these samples.

Of interest was the fact that all alligators appeared to have lymphoid lesions in some organ. Alligators in both the control and treatment group had lymphoid aggregates in the lungs, stomach and intestines, similar to the lesions observed in the skin. In addition, 38% (10/26; 95% CI: 20-58) of those alligators that had apparent skin lesions could not be corroborated on histopathologic exam. This may have been the result of sample processing or suggests that gross examination of the skin may be an inaccurate method of characterizing the LPSA status of alligators.

Table 6. Prevalence of lymphonodular lesions observed in tissues of 82 alligators.

| Tissue | Number of alligators with lesions | Prevalence | 95% Confidence Interval |
|----------------|--|-------------------|--------------------------------|
| Intestine | 76 | 93% | 87.5 – 98.5% |
| Lung | 64 | 78% | 69.1 – 86.9% |
| Stomach | 64 | 78% | 69.1 – 86.9% |
| Skin* | 36 | 44% | 33.2 – 54.8% |
| Kidney | 27 | 33% | 22.8 – 43.2% |
| Mesentery | 20 | 24% | 14.8 – 33.2% |
| Esophagus | 17 | 21% | 12.2 – 29.8% |
| Eye | 14 | 17% | 8.9 – 25.1% |
| Tonsil | 12 | 15% | 7.3 – 22.7% |
| Brain | 10 | 12% | 5.0 – 19.0% |
| Thyroid | 10 | 12% | 5.0 – 19.0% |
| Ovary | 9 | 11% | 4.2 – 17.8% |
| Conjunctiva | 8 | 10% | 3.5 – 16.5% |
| Heart | 7 | 9% | 2.8 – 15.2% |
| Testicle | 7 | 9% | 2.8 – 15.2% |
| Pancreas | 6 | 7% | 1.5 – 12.5% |
| Gall Bladder | 4 | 5% | 0.3 – 9.7% |
| Bone Marrow | 3 | 4% | 0.2 – 8.2% |
| Salivary gland | 1 | 1% | 0 – 3.2% |
| Spinal cord | 1 | 1% | 0 – 3.2% |
| Spleen | 1 | 1% | 0 – 3.2% |
| Ureter | 1 | 1% | 0 – 3.2% |

*Based on 81 skin samples

Table 7. Additional histopathologic changes recorded in 82 alligators

| Tissue | Lesion description | Number of animals with lesions | Prevalence | 95% Confidence Interval |
|---------------|---------------------------------------|---------------------------------------|-------------------|--------------------------------|
| Adrenal gland | Vacuolar changes/stress response | 36 | 44% | 33.3-54.7% |
| Liver | Vacuolar changes/unknown significance | 78 | 95% | 90.3-99.7% |
| Muscle | Rhabdomyolysis/stress response | 23 | 28% | 18.3-37.7% |

The histopathologic findings in the lungs and the intestinal tract (stomach and intestines) were novel. Reptiles do not have lymph nodes, and instead use aggregates of lymphoid tissues (e.g., Peyer's patches) to process antigens. One of our concerns was that some of these visceral sites might represent normal aggregates of lymphoid tissue and therefore contributed to the perceived lack of a control group. Additional tissue samples collected from four hatchling alligators showed similar lymphonodular responses in the stomach (3/4 hatchlings = 75%) and mesentery (1/4 hatchlings = 25%). The findings of the lymphoid infiltrates may represent areas

responsible for processing foreign antigens. When an antigen such as an allergen, virus, bacteria, or fungus enters the body, the intestines or lungs are likely to be the first sites of antigenic stimulation. The processing of these antigens, especially in the face of chronic stimulation, can lead to a significant cell-mediated and/or humoral immune response. The inflammatory response characterized in these alligators was consistent with those described in higher vertebrates.

Although the findings in the intestine and lungs may be associated with expected lymphoid aggregates, similar lesions in other organs not generally considered to be site specific for antigen processing suggest that the histopathologic findings are abnormal.

The histopathologic findings reported in this study suggest that a systemic disease process is occurring. More specifically, the findings were highly suggestive of an infectious disease process.

Electron Microscopy

Results of electron microscopy revealed flagellated protozoa (Figure 17) and an intracellular bacterium that lacked a cell wall and was consistent with *Mycoplasma sp.* (Figure 18). The protozoa could not be characterized any further. These results could not be duplicated.

Fungal Isolation

Fourteen different fungi were isolated from the skin of alligators during this project (Figure 19). These findings included multiple isolates from multiple animals as well as individual animals. Most of these fungi are ubiquitous in nature and are found in soil and water. Some of these fungi have been previously reported as causing secondary disease in reptiles (Tappe et al.1984, Jacobson 1979b, Cheatwood 2000).

Bacterial Isolation

Bacterial cultures revealed a mixed population of Gram-positive and Gram-negative organisms. No pure isolates were obtained. Histopathologic exam revealed acid-fast positive

bacilli, which were consistent with *Mycobacterium* species. However, this type of bacteria requires more specialized culture methods than were employed in this project.

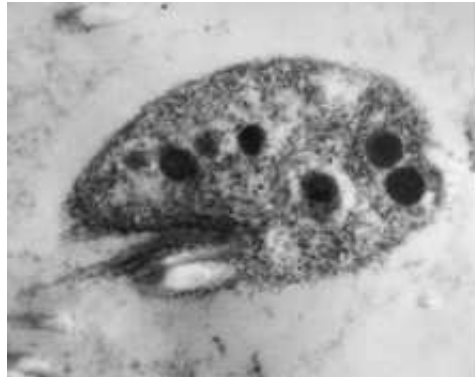


Figure 17. Transmission electron microscopic examination of a skin lesion from an alligator with mild LPSA revealed the above protozoa.

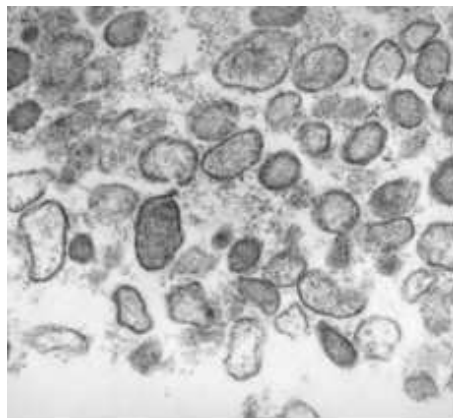


Figure 18. Transmission electron microscopic examination of a skin lesion from an alligator with mild LPSA revealed these bacteria. The bacteria identified in the EM did not have a cell wall.

Alternaria alternate
Aspergillus niger
Cladosporium cladosporioides, *Cladosporium oxysporum*
Chaetomium globosum, *Chaetomium spp.*
Curvularia lunata
Epicoccum purpurascens
Eurotium chevalieri
Monascus rubber
Mucor circinelloides (group)
Paecilomyces variotii
Penicillium citrinum
Syncephalastrum racemosum

Figure 19. Fungi isolated from the skin of captive American alligators.

Plasma Biochemistries

The results of the chemistry analytes are presented in table 8. The mean/median values for creatine kinase (CK) and aspartate aminotransferase (AST) were elevated, when compared to other reptile species. Both of these enzymes originate in the muscle and are likely an indicator of muscle necrosis, which can occur as a result of capture, restraint and venipuncture. A review of the other analytes suggests that there is no obvious physiologic disturbance to the plasma biochemistries, except for CK and AST, associated with the clinical and histopathologic findings of LPSA.

Protein Electrophoresis

When comparing our results with those reported in avian species, there are apparent changes in the protein electrophoresis results that may be of importance. Phylogenetically, birds are close relative to alligators and may prove valuable as a comparative species. The alligators in this study had decreased albumin-globulin ratios (A/G) and albumin levels, while the alpha- 2 proteins were increased (Tables 9 and10). The alpha – 1 and gamma protein fractions were on the high end of values observed in other reptile species. The changes in the protein electrophoresis observed in these alligators may be indicative of acute inflammatory disease.

Complete Blood Counts

Results of the CBC's are presented on table 11. Overall, the white blood cell count and cell differentials are consistent with a stress response and possibly an underlying inflammatory process. There are several outliers that had extremely elevated white blood cell and monocyte counts, but these might be expected in a population of animals that are held under intensive production operations. There is no evidence of a severe inflammatory response associated with LPSA.

Table 8. Plasma biochemistry values for 46 captive reared alligators

| Analyte | Mean/Median ¹ | SD ² | Minimum | Maximum | 95% CI ³ |
|--------------------------------|--------------------------|-----------------|---------|---------|---------------------|
| Glucose MG/DL | 110 | - | 83 | 244 | - |
| Aspartate Aminotransferase U/L | 261 | - | 162 | 589 | - |
| Creatine kinase U/L | 599 | - | 134 | 4337 | - |
| Tot. Protein G/Dl ^a | 5 | 1.36 | 4 | 7 | 5.2 – 5.6 |
| Albumin G/DL | 2 | - | 1 | 2 | - |
| Globulin G/Dl ^a | 4 | 1.14 | 3 | 5 | 3.6 – 4 |
| Calcium MG/Dl ^a | 12 | 1.3 | 10 | 13 | 11.5 – 11.9 |
| Phosphorus MG/Dl ^a | 5 | 1.84 | 4 | 8 | 5 – 6 |
| Sodium mmol/L | 151 | - | 89 | 162 | - |
| Potassium mmol/L ^a | 4 | 1.46 | 2 | 6 | 3.9 – 4.3 |
| Chloride mmol/L | 113 | - | 66 | 124 | - |
| TCO2 mmol/L ^a | 14 | 11.66 | 3 | 26 | 12 – 15 |
| Anion Gap mmol/L ^a | 28 | 18.04 | 12 | 51 | 25 – 30 |
| Uric Acid MG/DL | 1 | - | 0 | 4 | - |

¹Mean reported for normally distributed values, and median for non-normally distributed values, ²SD = two standard deviations, ³Confidence Interval (CI), ^aValues with normal distribution.

Table 9. Results of Protein Electrophoresis from 45 captive alligators

| Analyte | Mean/Median ¹ | SD ² | Minimum | Maximum | 95%CI ³ |
|----------------------|--------------------------|-----------------|---------|---------|--------------------|
| Total Protein | 5.3 | - | 3.4 | 7.6 | - |
| Albumin/Globulin | 0.2 | - | 0.2 | 0.2 | - |
| Albumin | 0.9 | - | 0.5 | 1.4 | - |
| Alpha 1 ^a | 0.6 | 0.6 | 0.2 | 2.2 | 0.5 - 0.6 |
| Alpha 2 | 2 | - | 0.8 | 3 | - |
| Beta | 1.1 | - | 0.7 | 1.6 | - |
| Gamma | 0.7 | - | 0.3 | 1.4 | - |

¹Mean reported for normally distributed values, and median for non-normally distributed values. ²SD = two standard deviations, ³Confidence Interval (CI), ^aValues with normal distribution

Table 10. Protein Electrophoresis Relative % Fractions from 50 *Alligator mississippiensis*.

| Fraction | Mean/Median ¹ | SD ² | Minimum | Maximum | 95% CI ² |
|----------------------|--------------------------|-----------------|---------|---------|---------------------|
| Albumin | 17.78 | - | 13.49 | 22.39 | - |
| Alpha 1 ^a | 10 | 0.6 | 4.07 | 13.8 | 8.71 - 10.0 |
| Alpha 2 | 38.01 | - | 34.67 | 42.66 | - |
| Beta | 20.42 | - | 15.85 | 25.12 | - |
| Gamma ^a | 13.49 | 0.5 | 5.01 | 22.91 | 12.59 - 14.13 |

¹Mean reported for normally distributed values, and median for non-normally distributed values. ²SD = two standard deviations, ³Confidence Interval (CI), ^aValues with normal distribution

Table 11. Complete Blood Counts from 45 alligators

| Value | Median | Min | Max |
|------------------------|---------------|------------|------------|
| Total White Cell Count | 7771 | 4098 | 27291 |
| Heterophils | 4118 | 1821 | 20741 |
| Lymphocytes | 818 | 0 | 2558 |
| Monocytes | 1324 | 366 | 3493 |
| Basophils | 828 | 0 | 2863 |
| Eosinophils | 609 | 100 | 1945 |

Water Analysis

Results of the water analysis are reported in table 12. There was no significant difference in the water chemistry parameters among farms (Table 12).

Table 12. Water analysis values from alligator farms (N=11 samples from 6 farms)

| Analyte | Median | Minimum | Maximum | p Value¹ |
|------------------------|---------------|----------------|----------------|----------------------------|
| pH | 7.57 | 6.86 | 8.33 | 0.2 |
| Ammonia (ppm) | 30.86 | 0.46 | 171 | 0.71 |
| Bromide (ppm) | 0.4 | 0.2 | 1.2 | 0.08 |
| Fluoride (ppm) | 1.83 | 0.53 | 9.67 | 0.44 |
| Nitrate (ppm) | 1.59 | 0.3 | 6.49 | 0.7 |
| Phosphate (ppm) | 22.12 | 1 | 97.3 | 0.7 |
| Sulphate (ppm) | 36.59 | 1.26 | 138 | 0.27 |
| Total solids (ppm) | 1094.72 | 446 | 2242 | 0.68 |
| Suspended solids (ppm) | 259.31 | 20 | 881 | 0.22 |
| Dissolved solids (ppm) | 837.23 | 427 | 1593 | 0.23 |

¹ p value for comparison of each parameter between farms

Heavy Metal and Trace Elements Screening

Blood and tissue concentrations for copper, iron, lead, mercury, selenium, and zinc are reported in table 13. These values approximate levels previously reported from crocodylians (Burger et al. 2000, Jeffree et al. 2001).

Herpes Virus PCR

We were unable to identify any herpes viruses from the tissues of alligators. A herpes simplex one virus was identified but determined to be a contaminant since the laboratory where the work was performed routinely works with herpes simplex. Attempts to minimize contamination, and re-evaluation of reagents and procedures were performed in order to identify

problems with the PCR assay. Despite these attempts and procedure modifications, no herpes virus was identified.

Table 13. Heavy Metals and Trace Elements Levels from Blood and Tissues of Alligators.

| Test | Tissue | N | Mean/Median ¹ | SD ² | Min | Max | 95% CI ³ |
|--------------|--------|----|--------------------------|-----------------|------|------|---------------------|
| Copper ppm | Blood | 50 | 0.6 | - | 0.4 | 0.9 | - |
| | Kidney | 14 | 2.1 | - | 1.4 | 5.5 | - |
| | Liver | 50 | 7.2 | - | 3 | 46 | - |
| | Muscle | 50 | 0.14 | - | 0.09 | 0.52 | - |
| Iron ppm | Blood | 50 | 1.1 ^a | 0.74 | 0.26 | 2 | 0.98 - 1.19 |
| | Kidney | 14 | 43.2 ^a | 23.44 | 27.1 | 67.3 | 36.43 - 49.97 |
| | Liver | 50 | 118.7 | - | 23 | 1892 | - |
| | Muscle | 50 | 3.75 | - | 1.9 | 19 | - |
| Lead ppm | Blood | 14 | 0.02 | - | 0.02 | 0.14 | - |
| | Kidney | 13 | 0.03 | - | 0.02 | 0.28 | - |
| | Liver | 14 | 0.02 | - | 0.02 | 0.04 | - |
| | Muscle | 14 | 0.02 | - | 0.02 | 0.04 | - |
| Mercury ppm | Blood | 14 | 0.02 | - | 0.01 | 0.09 | - |
| | Kidney | 13 | 0.02 | - | 0.01 | 0.16 | - |
| | Liver | 14 | 0.02 | - | 0.01 | 0.1 | - |
| | Muscle | 14 | 0.01 | - | 0.01 | 0.03 | - |
| Zinc ppm | Blood | 50 | 0.4 | - | 0.2 | 0.6 | - |
| | Kidney | 14 | 14.44 ^a | 13.22 | 10.7 | 17.2 | 13.37 - 15.50 |
| | Liver | 50 | 12.8 | - | 9.8 | 21.7 | - |
| | Muscle | 50 | 13.6 | - | 5.5 | 23.7 | - |
| Selenium ppm | Blood | 50 | 0.24 | - | 0.16 | 0.4 | - |
| | Kidney | 14 | 0.78 ^a | 0.3 | 0.53 | 1.02 | 0.69 - 0.87 |
| | Liver | 50 | 0.58 | - | 0.4 | 1.16 | - |
| | Muscle | 50 | 0.2 ^a | 0.08 | 0.13 | 0.29 | 0.19 - 0.21 |

¹Mean reported for normally distributed values, and median for non-normally distributed values. ²SD = two standard deviations, ³Confidence Interval (CI), ^aValues with normal distribution

Discussion

A general deficiency of this study was that the perception that there may have been no obvious control group. Instead, descriptive statistics were used to characterize the results and evaluate the general health of all alligators tested. The results suggest that there are mild underlying changes in these alligators, but that more information is needed to interpret these results. Attempts to identify control animals and compare results collected from those animals retrospectively with this data will be pursued.

From these descriptive results, it is apparent that these alligators are producing a systemic inflammatory response. Although LPSA is generally considered a disease of the skin, it is apparently more than that. Attempts to characterize the degree of normality associated with the lymphohistiocytic proliferative syndrome in the intestines and lungs must be done. This will be further evaluated when the control alligators are found and processed. Because of the economic importance of LPSA, it is imperative that attempts to classify a specific etiology are pursued.

The histopathologic findings were crucial to help determine which disease etiology (-ies) may be more likely associated with LPSA. The findings of the electron microscopy were not duplicable and were ultimately interpreted to be incidental findings with no association to the pathology observed in the tissues. Besides the incidental finding on EM, parasitic organisms were not observed in any of the LPSA lesions. Of great importance is the lack of histologic evidence of a fungal infection in LPSA tissues. For this reason we believe that there is no evidence to indicate that fungi are the causative agents of LPSA in Louisiana. The results of the fungal culture were not unexpected, as a significant amount of fungal organisms are ubiquitous and can normally be identified using culture techniques. What was interesting was the wide variety of fungi that was isolated. These data can provide a reference of fungal organisms that can be found in alligator farms in Louisiana. If primary fungal infections in captive alligators were to be diagnosed, one of these fungi may be responsible for such infections. Finally, we must look back at the cell types present within the LPSA lesions. Lymphohistiocytic inflammation is more commonly associated with a viral diseases or an immune mediated disease. In light of the information presented and the lack of evidence to support a bacterial or fungal etiology, a virus remains as the most likely cause of LPSA.

An interesting finding was the identification of acid fast positive organisms consistent with *Mycobacterium spp.*. However, histopathologic evaluation of these tissues was not

consistent with expected changes for a mycobacterium infection making it a likely incidental finding. There is also the possibility of those organisms being a different acid fast bacteria other than *Mycobacterium sp.*, such as *Nocardia sp.*. There was no histologic association observed between LPSA lesions and the acid fast organisms. Furthermore no bacterial organisms of any type were observed within LPSA lesions, making a bacterial etiology less likely for LPSA.

The findings of the CBC suggest a lack of a significant inflammatory response despite the LPSA lesions. A possible explanation for this finding is that alligators with LPSA in the early or late stages may not have mobilized significant cell numbers or may be past the major cell migration. The observation that many of the lesions in the tissues were mild may support an early state of disease. The combination of the histopathologic lesions, protein electrophoresis and CBC results suggest that there is evidence of a low-grade inflammatory response underlying the stress response. To further characterize the suspected inflammatory response, control alligators or serial samples from the same affected alligator would be required.

Heavy metal values were consistent with values previously reported in crocodylians (Burger et al. 2000, Jeffree et al. 2001). Because these values were similar to those previously reported, it is unlikely that these compounds play an important role in the development of LPSA. However, toxins can play a secondary role in disease processes, and should never be ruled out based only on a comparative reference. The results for the various water chemistries reveal higher levels of ammonia and solids than what would be acceptable for fish aquaculture (Brune and Tomasso 1991, Noga 2000). These higher values are not surprising due to the management techniques employed in alligator facilities, the high protein content of the alligator diet, and the large amount of fecal matter produced. The lack of difference between farms and the fact that these levels are typical of what is observed in facilities without LPSA, make water quality an unlikely contributor to the development of LPSA.

During this study it became evident that LPSA was more widespread than previously imagined. Although this syndrome appears to have only been identified in 2002, it is possible that the disease was present before that time and went unnoticed. One would suspect that it would be identified at the tanner, but if the lesions were less common, they may have been missed. Attempts to evaluate leather processed prior to 2002 for LPSA should be done. One interesting observation about the occurrence of LPSA is that it appeared to match the occurrence of WNV which was first observed in 2001 in Louisiana. Based on the results of this study, particularly the histopathologic evaluation of LPSA tissues, a viral etiology remains as the top differential for LPSA.

CHAPTER 2: WEST NILE VIRUS IN ALLIGATOR, *ALLIGATOR MISSISSIPPIENSIS*, RANCHES FROM LOUISIANA*

Introduction

West Nile virus (WNV) has been reported to affect various crocodylian species including the American alligator, *Alligator mississippiensis*, (Miller et al. 2003), the Nile crocodile, *Crocodylus niloticus*, (Steinman et al. 2003), and the Morelet's crocodile, *Crocodylus moreletii*, (Rubio 2004). The role of crocodylians and other reptiles in the epidemiology of WNV infection is largely unknown. However there is evidence of high viremias and viral shedding in crocodylian species, specifically the American alligator, based on published articles and our clinical experience (Miller et al. 2003). If crocodylians are able to maintain high viral loads, they can serve as an amplifying host. This creates a potential public health problem in captive operations where humans may be exposed to WNV by direct contact with the animal's feces and tissues. There have been three cases of suspected WNV infection in humans working in an alligator ranch in Louisiana. In addition there was one confirmed case of WNV exposure in Idaho based on antibody titers (Tengelsen 2004). This individual had handled tissues from WNV positive alligators but did not report clinical signs.

Florida, Georgia, Louisiana, and Idaho have all experienced cases of WNV in alligators. This case report presents our clinical experience with diagnosis of WNV in four alligator ranches from Louisiana. The first suspect cases in Louisiana occurred in October of 2003. All cases were observed between October and December of 2003. Of the ranches in Louisiana, two stocked imported hatchlings from Florida, one from Texas, and the fourth stocked locally hatched alligators.

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Materials and Methods

All hatchlings were housed indoors with water temperatures between 29 – 32°C (85 – 90°F). Water sources varied between ranches and included city water, well water, or bayou water. Water changes were performed either daily or every other day. Some farms added bleach at an unknown concentration with each water change. Stocking densities varied but were in accordance with the recommendations from the Louisiana Department of Wildlife and Fisheries. These recommendations are as follows: one square foot per animal for alligators smaller than twenty four inches in length, three square feet per animal for alligators measuring twenty five to forty eight inches, for every six inches in length beyond forty eight inches you should add one square foot of space (Elsey 2004). Feed consisted of a commercial pelleted alligator ration. There was no exposure to meat sources such as beef, horse meat, pork, or nutria. One ranch occasionally supplemented with chicken or fish. There was no exposure to wild birds inside the buildings. However, mosquitoes were reported to be present inside the building on a regular basis.

The initial clinical signs were observed approximately seven to ten days after the imported animals arrived at the respective facilities. These animals were imported at approximately 20 to 30 d old. The locally harvested alligators ranged in age from two months to fourteen months. Clinical signs varied from peracute death to a variety of neurological manifestations. Swimming in circles, ataxia, head and muscle tremors, and head tilt were observed in the affected alligators (Figure 20). Stomachs were void of ingesta, but were full of water at necropsy, suggesting that these animals were anorectic. The large amount of water in the stomach may also suggest that the basihyoid and palatine valves were not working properly, perhaps due to weakness associated with the disease. A less common sign was bloating, as evidenced by the alligator's inability to submerge

properly under the water. The caudal aspect of the body remained afloat, sometimes sideways, while the cranial aspect of the body was observed underwater. Necropsy of these animals revealed a colitis characterized by formation of a fibrinous membrane on the mucosa (Figure 21).



Figure 20. American Alligator with a head tilt as consequence of WNV infection.

This last presentation was primarily observed in the yearling alligators (12 – 14 months old). Mortalities ranged from 40 – 60% of alligators within an affected pen or building with an estimate of over 5,000 deaths in the whole state. In most instances, mortalities were swift in one to two month old animals with deaths occurring suddenly within a week of onset of clinical signs. This was followed by an equally fast decline in the number of deaths. On the other hand, mortalities in yearling animals followed a more sporadic course and spread over weeks or even months.

For example, a building housing 5 wk old alligators may have experienced 50 or 60 mortalities a day, while in yearling alligators the deaths rarely surpassed 15 – 20 animals

per day. Mortalities were primarily confined to a single pen or building. Once a peak in mortalities occurred, the disease appeared to dissipate and remaining animals continued to



Figure 21. Fibrinous membrane on the colon mucosa of a WNV positive alligator. WNV was identified from this lesion via RTPCR, viral isolation, and immunohistochemistry.

thrive without evidence of illness. However, in one particular case, an alligator rancher reported mortalities of alligators spreading through the facility. After obtaining a detailed history of events preceding the deaths, it was evident that the mortalities followed a pattern of animal movement between buildings. The owner had been instructed not to move alligators from affected buildings; however, because of size constraints, animals were moved from affected buildings into non-affected buildings. The mortalities in the new buildings followed in chronology the addition of new animals previously housed in buildings with WNV positive alligators. The first deaths were observed approximately seven to ten days after the introduction of affected alligators. By the time we confirmed WNV infection, mortalities were identified in all of the buildings into which the previously exposed animals were introduced. Although this spread of WNV was only identified in one ranch, it reinforces the importance of increased biosecurity in order to minimize the spread of WNV between captive alligators.

The clinical approach for these cases consisted of a visit to the facilities in order to observe and collect affected animals. Necropsies were performed using sterile techniques. All tissues were collected from fresh specimens. Brain was collected from all animals and submitted for either real time RT-PCR, viral isolation, or both. Thirteen brain samples were submitted for real time RT-PCR and viral isolation. An additional nine samples were submitted for viral isolation alone. A sample of colon from an alligator displaying clinical signs of bloating with fibrinous colitis was also submitted for real time RT-PCR and viral isolation. A second colon sample was submitted for viral isolation only. One fecal sample was submitted for viral isolation. Histopathologic examination was performed on fourteen alligators.

RNA Extraction and Real Time RT-PCR

The RNA was extracted from tissue using the Qiagen Rneasy kit (Qiagen, 27220 Turnberry Lane Suite 200, Valencia, CA) following the manufacturer's protocol. Approximately 20 mg of tissue was homogenized in 350 μ l of Rneasy lysis buffer in a Retsch Laboratory Vibration Mill Type MM 300 with a copper BB at 25 cycles per second for 5 min (Retsch Inc., 74 Walker Lane, Newtown, PA). The homogenized samples were subjected to Rneasy extraction and eluted in 30 μ l of Rnase-free water. A primer-probe set targeting the WNV envelope gene was used for the real time RT-PCR (Lanciotti et al. 2000). The probe contained a 5' reporter 6-carboxyfluorescein (FAM) and a BHQ-1 quencher as reported by Lanciotti (Lanciotti et al. 2000). The assay was performed on an ABI 7900 Taqman Sequence Detector (Applied Biosystems, 850 Lincoln Centre Dr, Foster City, CA) with Quantitec Probe RT-PCR master mixture (Qiagen, 27220 Turnberry Lane Suite 200, Valencia, CA). The reaction mixture contained 50 picomoles of each primer, 10 picomoles of the probe and 5 μ l of RNA elute in a total volume of 50 μ l. The

reaction conditions were: 30 min at 48°C (118°F) for RT, 15 min at 95°C (203°F) to activate the Taq, and then 45 cycles of 15 sec at 95°C (203°F) and one minute at 60°C (140°F).

Viral Isolation

For viral culture attempts, a 10 – 15% tissue suspension was made using a stomacher 80 homogenizer (Seward LTD., London, UK) in an isotonic solution containing 0.22 M sucrose, buffered with 0.01M potassium phosphate, and containing 0.1 mg/ml gentamicin (Sigma Laboratories, St. Louis, MO), 0.5 mg/ml kanamycin (Sigma Laboratories, St. Louis, MO), and 0.1 mg/ml vancomycin (Sigma Laboratories, St. Louis, MO) and 0.01 mg/ml amphotericin B (Sigma Laboratories, St. Louis, MO). The suspensions were refrigerated overnight, and then clarified by centrifugation at 2000 x g for 30 min at 10°C (50°F). The cell culture growth medium consisted of Modified Eagle's Medium (MEM) with 2.2 g/L of bicarbonate and 2.0 mM L-glutamine (Hyclone, Logan, UT), 25 mM HEPES buffer (Sigma Laboratories, St. Louis, MO), and 5% fetal bovine serum (Hyclone, Logan, UT) and was removed from one day old sub-confluent Vero cell line grown on cover slips in 1 dram, 15 x 45 mm, shell vials (03-339-26B, Fisher Scientific, Pittsburg, PA) (Lennette et al. 1999). Each clarified tissue suspension was inoculated into a separate set of shell vials in volumes of 0.2 ml per vial. The inoculated shell vials were incubated at 36°C (96.8°F) for 90 min. The inocula were removed from the shell vials, which were then rinsed and replenished with MEM as in the cell culture growth medium composition mentioned before but containing 1% fetal bovine serum, 200 U/mL of penicillin (Sigma Laboratories, St. Louis, MO), and 0.2 mg/mL of streptomycin (Sigma Laboratories, St. Louis, MO). The vials were incubated at 36°C (96.8°F) and examined daily for evidence of cytopathic effect (CPE). At seven days post-inoculation (PI), material

was scraped from the bottom of one vial of each inoculated set and sub-cultured onto a fresh set of Vero shell vials. If there was no CPE evident by three days PI on the sub-culture (ten days PI from the original inoculation), then the cover slips were stained with the Giemsa method and examined at 100X for microscopic evidence of cytopathic effects (CPE). Negative cultures were completed at this point, and suspicious cultures were sub-cultured again. If CPE appeared during the course of incubation, material was sub-cultured onto chamber slides (Lab-Tek Chamber Slides, Nalge Nunc International, Naperville, IL) containing sub-confluent Vero cell line as described above. The slides were incubated for two to three days to allow CPE to develop, and then were rinsed, acetone-fixed, and stained with a two-step, indirect immunofluorescent method using commercially-available anti arboviral monoclonal antibodies (Chemicon International, Inc, Temecula, CA) and FITC conjugated anti-mouse antibody (KPL, Gaithersburg, MD). These were reactive to West Nile virus, St. Louis encephalitis virus, and eastern equine encephalitis virus, as well as western equine encephalitis virus.

Histopathology

Tissue specimens were fixed in 10% neutral buffered neutral formalin overnight, trimmed, processed, sectioned, and stained with hematoxylin and eosin (H&E) for microscopic examination. For immunohistochemistry, a two-step, non-avidin-biotin method (EnVision®+, Dako Cytomation California Inc., 6392 Via Real, Carpinteria, CA) was performed. Formalin-fixed, paraffin-embedded tissues were sectioned by 4 µm, placed on positive-charged slides, and pretreated with proteinase K (Dako Cytomation California Inc.) for ten minutes. The staining procedures were carried out according to the manufacturer's recommendation. Mouse anti-WNV monoclonal antibody (ATCC, Manassas, VA) was used as primary antibody. Normal mouse serum replaced the primary

antibody for negative control. Visualization was achieved by using Nova Red[®] (Vector Laboratories, Burlingame, CA). H&E stain was used for counter-staining.

Results

Real time RT-PCR was a reliable test for diagnosing WNV from the brain tissue of alligators (Table 14). All of the samples submitted for real time RT-PCR (13/13; 100%) were positive for WNV. Of these thirteen samples, ten (77%, 95% CI: 54-100%) were also positive via viral isolation. Seven out of nine additional samples (78%, 95% CI: 51-100%) submitted for viral isolation only also tested positive for WNV (Table 14). These results add up to a total of seventeen positive cases diagnosed by viral isolation (77%, 95% CI: 59-95%). For the colon samples; one was positive via both real time RT-PCR and viral isolation, while a second sample submitted only for viral isolation also tested positive.

Positive WNV cultures typically exhibited viral CPE before the end of the sub-culture period. Certain isolates displayed CPE earlier in the course of culture (three to five days PI during the primary culture period) presumably because the original tissue from these alligators contained a relatively high amount of infectious virus. All WNV positive cultures displayed immunofluorescence when stained with WNV and flavivirus-specific monoclonals (Figure 22). Cultures and controls were negative when stained with monoclonals against St Louis Encephalitis (SLE), Eastern Equine Encephalitis (EEE), and Western Equine Encephalitis (WEE) viruses. Negative samples and controls failed to develop viral CPE. Selected CPE-negative cultures failed to reveal fluorescence when stained with monoclonals as described above. RNA from culture material of one positive WNV alligator isolate was extracted, analyzed and verified as WNV by RT-PCR. A nucleotide segment of approximately 250 base pairs in length was produced using a nested

Table 14. Results of WNV RT-PCR and viral isolation from 22 American alligators in Louisiana.

| Ranch | N | Alligator | Tissue | RT-PCR | Viral Isolation |
|-------|---|-----------|--------|----------|-----------------|
| A | 5 | 1 | Brain | | positive |
| | | 2 | Brain | | positive |
| | | 3 | Brain | | positive |
| | | 4 | Brain | | negative |
| | | 5 | Brain | | positive |
| B | 4 | 1 | Brain | positive | positive |
| | | 2 | Brain | positive | positive |
| | | 3 | Brain | positive | positive |
| | | 4 | Brain | positive | positive |
| C | 4 | 1 | Brain | positive | positive |
| | | 2 | Brain | | positive |
| | | 3 | Brain | | positive |
| | | 4 | Brain | positive | positive |
| D | 9 | 1 | Brain | positive | positive |
| | | 2 | Brain | positive | negative |
| | | 3 | Brain | | negative |
| | | | Colon | | positive |
| | | | Feces | | negative |
| | | 4 | Brain | | positive |
| | | | Colon | positive | positive |
| | | 5 | Brain | positive | negative |
| | | 6 | Brain | positive | negative |
| | | 7 | Brain | positive | positive |
| | | 8 | Brain | positive | positive |
| | | 9 | Brain | positive | positive |

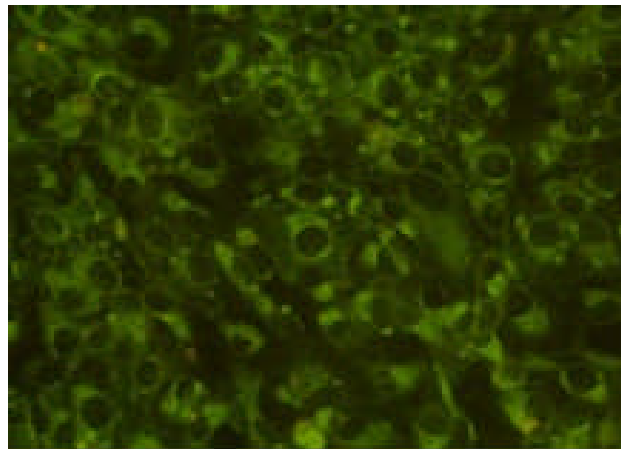


Figure 22. Image of WNV infected Vero cells with stained fluorescent antibodies against flavivirus envelope glycoprotein shown as green (100X).

RT-PCR procedure as for diagnostic testing (Johnson et al. 2001), which targeted a conserved region partially coding for the WNV envelope glycoprotein. The segment was sequenced to demonstrate homology (approximately 95%) with a published sequence of the corresponding portion of the WNV genome (GenBank accession number AF196835).

Microscopically, all alligators had similar lesions but differed in severity (Table 15). The common lesions were diffuse severe heterophilic, histiocytic, and necrotizing enterocolitis, heterophilic meningoencephalitis, necrotizing and heterophilic hepatitis, heterophilic and histiocytic splenitis, generalized heterophilic and histiocytic lymphoid folliculitis, and necrotizing and heterophilic pancreatitis. Less common were mild multifocal heterophilic and lymphohistiocytic interstitial nephritis, gastritis, and mild pulmonary congestion and edema. In addition, immunohistochemistry for WNV revealed strong immunopositivity in the brain, liver, spleen, pancreas, kidney, and gastrointestinal tract of all animals that tested positive for WNV via RT-PCR and/or viral isolation (Figure 23).

Discussion

The early recognition of clinical signs is essential in the diagnosis of WNV in alligators. Because affected animals developed a consistent array of neurological signs, WNV should be high in a differential list for alligators with neurological diseases. All suspected cases in which viral tests were performed were positively diagnosed by RT-PCR. Two cases in which viral isolation was attempted were negative. Immunohistochemistry may also prove to be a useful test with potential for ante-mortem diagnosis.

The early appearance of CPE in many, but not all, of the alligator isolates, relative to our experiences with avian and equine isolates, suggests that either alligator field

Table 15. Histopathologic Findings in 14 alligators diagnosed with WNV in Louisiana.

| Tissue | Lesion | Prevalence | 95% CI ¹ |
|-----------|-------------------------------------|------------|---------------------|
| Brain | Heterophilic meningoencephalitis | 100% | - |
| Intestine | Heterophilic enterocolitis | 35.7% | 10.7-60.7% |
| Liver | Heterophilic /histiocytic hepatitis | 35.7% | 10.7-60.7% |
| Lungs | Heterophilic inflammation | 35.7% | 10.7-60.7% |
| Stomach | Heterophilic gastritis | 28.6% | 4.9-52.3% |
| Pancreas | Heterophilic pancreatitis | 21.4% | 0.1-42.7% |
| | Coagulative necrosis | 14.3% | 0-32.5% |
| Spleen | Heterophilic splenitis | 21.4% | 0.1-42.7% |
| Kidney | Heterophilic nephritis | 14.3% | 0-32.5% |
| Thymus | Heterophilic inflammation | 14.3% | 0-32.5% |
| Heart | Heterophilic inflammation | 14.3% | 0-32.5% |
| Ovaries | Heterophilic inflammation | 7.1% | 0-20.5% |

¹95% Confidence Interval

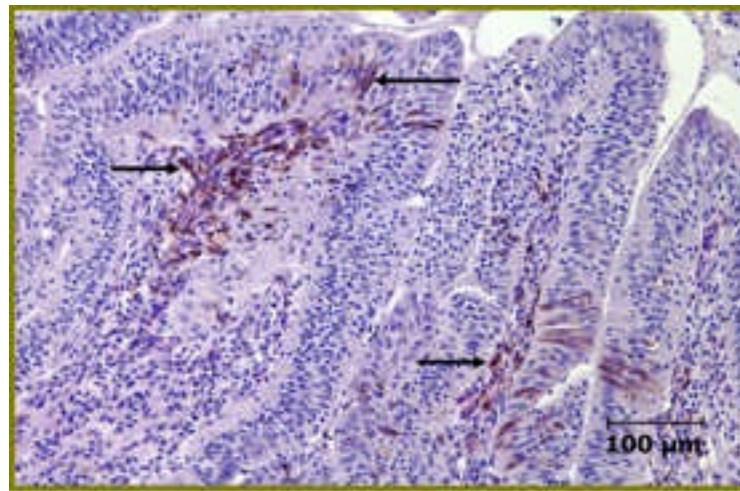


Figure 23. Colon of an alligator showing positive immunohistochemistry for WNV. Black arrows show immunopositive stained cells (100X).

strains are able to adapt and grow more readily than other WNV strains in the Vero cell line, or that the original tissue samples contained an inordinately high amount of infectious virus, relative to tissues from other naturally-infected species. So far we have no evidence that WNV strains in alligators differ from those in other species in Louisiana. The early CPE was judged to be dose dependent, rather than due to adaptability, because all alligator isolates failed to exhibit uniformly early CPE. WNV

may be able to grow in the tissues of immunologically naive alligators with greater efficiency than in tissues of other species. This possibility, along with observations that most alligators within affected habitats displayed evidence of infection, and that WNV was recovered by culture from nearly all alligators sampled in affected ranches, suggests that WNV may be spread in alligators by additional, more aggressive modes of transmission, rather than vector transmission alone. Parenteral and oral experimental infection of alligators with WNV has been recently described (Klenk et al.2004.)

We believe that horizontal transmission plays a key role in the spread of WNV in alligators. This is based on our clinical observations and the positive identification of WNV from the intestines. Because only one of the affected facilities supplemented with chicken or fish, the possibility of infection occurring via the feeding of meat products, such as horse meat, is not likely in our scenario. Although the pelleted diets were not tested for WNV, not all facilities used the same brand of feed making this an unlikely source. Therefore, a bite by an infected mosquito remains the original route by which these alligators must have been infected. Afterwards, the rest of the animals in the pen or building may become infected by being in close contact with the WNV positive animals. The question still remains as to whether the imported hatchlings became infected in Louisiana or were they already infected at their original location in Texas or Florida. We do not have enough evidence to answer this. However we know that the alligator hatchlings imported from Florida were obtained from the same Florida facility that exported hatchlings to an alligator ranch in Idaho that same year. The hatchlings in Idaho were also diagnosed WNV positive. At that time Idaho had no reported cases of WNV. Therefore there appears to be a link between ranches in Louisiana and the ranch in Idaho as they obtained hatchlings diagnosed WNV positive from the same source in Florida.

As previously mentioned, one ranch continued to have mortalities associated with WNV after movement of infected animals. The mortalities in the buildings to which the animals were moved surpassed the amount of affected animals that were introduced. The mortalities were usually observed at seven to ten days after the animals had been moved. At the time of animal's movements, the rancher had already instituted an aggressive mosquito control program in conjunction with the local mosquito control and abatement unit. The numbers of mosquitoes were significantly less when the animals were moved. Interestingly, this facility is also the one in which the three suspect human cases of WNV were found. Two of the three individuals lived and worked in the ranch. The third individual worked in the ranch. All of them were active in the slaughter and processing of alligators. Although this is only one site, it provided us with an abundance of information into the way that WNV can behave in these facilities. It also provided evidence of the zoonotic risk of handling alligators infected with WNV and/or working in the affected facilities.

CHAPTER 3: ESTABLISHING AN ASSOCIATION BETWEEN WEST NILE VIRUS SEROPOSITIVITY AND THE DEVELOPMENT OF LYMPHOHISTIOCYTIC PROLIFERATIVE SYNDROME OF ALLIGATORS*

Introduction

Lymphohistiocytic Proliferative Syndrome of Alligators (LPSA) was first described in captive reared American alligators (*Alligator mississippiensis*) from Florida and Louisiana in 1999 and 2002, respectively (Dickson et al. 2001, Cardeilhac et al. 2001, Nevarez et al. 2003a). This syndrome is of primary interest to the alligator industry because it affects the quality of the alligator hide, resulting in significant financial losses for the alligator producer. Gross findings are generally characterized by the presence of 1-2mm, gray to red foci on the ventral scales of the abdomen, tail, and mandible (Figure 24). Lesions have not been identified on the dorsal scales, but this is likely due to the dark pigmentation of the skin in this area. Although the skin is the most common site for identifying gross lesions, microscopic evaluation of affected animals consistently reveals lymphocytic-histiocytic infiltrates in the intestines, lungs, stomach, and skin (Nevarez et al 2003b). The lymphocytic-histiocytic infiltrates have also been identified in other

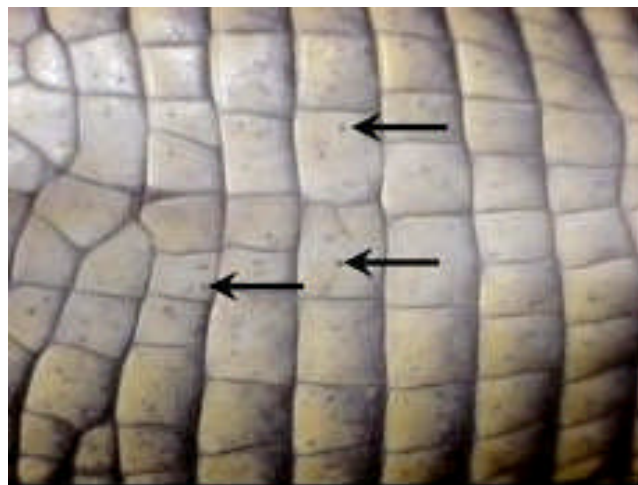


Figure 24. LPSA lesions (black arrows).

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tissues although with less frequency (Nevarez et al 2003b). In alligators, an increased number of infiltrates in the intestines and lungs may in part be due to normal aggregates of lymphoid tissue that are similar to the gut associated lymphoid tissue of mammals. However, the presence of these infiltrates in other non-gastrointestinal and respiratory tissues (e.g., skin, eye, brain) was not expected and suggests the findings represent a systemic response to a foreign material.

Since originally being described in Louisiana in 2002, regional LPSA epizootics continue to be reported in alligator farms in the state. The diagnosis of LPSA is generally made by the farmers at the time of slaughter or by individuals grading the hides at the leather processing plants. Discussions with alligator farmers from Louisiana have produced an interesting association between the development of LPSA lesions in their animals and the diagnosis of West Nile virus (WNV) on their farms. Alligators removed from a pen or building where WNV was diagnosed were, anecdotally, more likely to have LPSA at the time of slaughter, whereas animals from buildings where WNV was not diagnosed were not likely to have skin lesions. In the past, characterizing an association between the development of LPSA and WNV exposure was difficult because of limited diagnostic test availability. However, an indirect enzyme-linked immunoassay (ELISA) to detect WNV exposure in alligators is now available (Jacobson et al 2005). The purpose of this study was to determine if there is an association between the development of LPSA lesions in alligators and WNV exposure. The specific hypothesis being tested in this study was that LPSA positive alligators would be more likely to be seropositive for WNV than LPSA negative alligators.

Materials and Methods

Three Louisiana alligator farms with a history of LPSA-positive alligators were enrolled in the study between December, 2004 and March, 2006. Farm A was located in south Louisiana and raised approximately 50,000 alligators annually; farm B was located in central Louisiana and

raised approximately 13,000 alligators annually; farm C was located in northern Louisiana and raised approximately 30,000 alligators annually. WNV was diagnosed on farms A and B in the fall of 2004 and on farm C in the fall of 2005. The alligator farmers were asked to identify a building on their property with a history of animals with LPSA and another building in which LPSA animals had not been observed. Ten alligators destined for slaughter were collected from each building on each farm. The presence or absence of LPSA lesions on the skin was confirmed by both direct visualization of the integument on the live animal as well as observation of the hide on a light table after euthanasia. A single individual (JGN) with extensive experience diagnosing LPSA evaluated all of the alligator hides. A 3-ml blood sample was collected from the supravertebral sinus of each alligator ante-mortem. The blood samples were stored on frozen gel packs and transported to the Louisiana State University School of Veterinary Medicine for processing. The samples were centrifuged at 3,400 rpm for 10 minutes. The serum was removed and stored at -70°C until transported on frozen gel packs to the University of Florida College of Veterinary Medicine (Gainesville, FL 32608 USA) for serologic testing. An indirect ELISA validated for the detection of antibodies to WNV in American alligators was used to determine WNV exposure (Jacobson et al. 2005). A single-blind study was performed to minimize the potential for bias in interpreting the results of the ELISA.

A crude odds ratio (OR) was calculated to determine if there was an association between LPSA status and WNV serostatus of the alligators for all three farms. A Mantel-Haenszel (MH) adjusted OR was calculated by stratifying on farm to determine if there was a farm effect. If the difference between the crude and adjusted OR was >30%, then the adjusted OR was accepted. A MH χ^2 test was calculated to test the original hypothesis. A $p < 0.05$ was used to determine significance.

Results

All of the alligators from farms A, B and C with LPSA skin lesions were seropositive for WNV (30/30), while all the LPSA negative alligators were seronegative (30/30). After adjusting by farm, the MH adjusted OR revealed that LPSA positive alligators were 476 (95% CI: 79.6, 2845.2) times more likely to be seropositive for WNV than LPSA negative alligators. Because the difference between the crude and adjusted OR's was >30%, the adjusted OR is accepted. Alligators with LPSA lesions were significantly more likely to be WNV seropositive than LPSA negative alligators (MH χ^2 : 45.67, $p < 0.005$).

Discussion

LPSA is an inflammatory disease that is characterized by the presence of lymphocytes and histiocytes (lymphohistiocytic). These cells serve many different roles in vertebrates, including the induction and expression of the cellular and humoral immune response, phagocytosis and antigen presentation. This type of inflammatory response is primarily associated with viral infection, and has been observed in WNV cases with geese (Swayne et al. 2001), fox squirrels (Kiupel et al. 2003), and reindeer (Palmer et al. 2004). Prior work with LPSA has ruled out a bacterial or fungal etiology (Nevarez et al. 2003b). Herpes viruses are also known for creating lymphohistiocytic inflammation, but we were unable to find evidence of herpes virus via PCR (Nevarez et al. 2005a) or electron microscopy in animals with LPSA (Nevarez et al. 2003b). The results of this study suggest that there is a very strong association between LPSA and WNV serostatus.

In all three farms, the LPSA skin lesions appeared after WNV infection was diagnosed. The findings of this study suggest that seropositive alligators are 476 times more likely to develop LPSA skin lesions than those with no seroconversion. The lymphohistiocytic lesions found in LPSA animals may prove to be a chronic manifestation of WNV in alligators, as

compared to the heterophilic inflammation that is observed in acute cases. WNV has been actively diagnosed in the state of Louisiana since the fall of 2003 (Nevarez et al. 2005b). Before this date WNV was not considered an important disease of alligators and the absence of related diagnostic tests made it difficult to identify exposure. In 2005, there were three confirmed cases of WNV in Louisiana alligator farms, and all three farms reported LPSA skin lesions as early as 30 days post diagnosis of WNV. In the author's experience, the epidemiology of WNV in Louisiana has matched the occurrence of LPSA in alligator farms. In each confirmed case of WNV on a farm, the infection was isolated to one or two buildings, and the dissemination of the disease was minimized or prevented by strict quarantine. This was also found for LPSA outbreaks.

The findings of this study raise important questions regarding the epidemiology of WNV in the United States. PIX disease, a skin disease first described in Florida in 1999, is histologically similar to LPSA (Cardeilhac et al. 2001, Nevarez et al. 2003a, Nevarez et al. 2003b). If LPSA (and PIX disease) are the chronic manifestation of WNV in alligators, then WNV was potentially circulating in Florida in 1999, the same time it was first diagnosed in New York City, New York (CDC 1999). This suggests that WNV was not introduced into the United States in 1999, but at least one year earlier. This would be consistent with the epidemiology of other arboviruses, with the disease not being diagnosed until sufficient spillover occurred. It might be expected that WNV could be disseminated between New York City and Florida via bird migration. Based on these assumptions, it is also possible that WNV was first introduced in Florida, and that the 1999 outbreak in New York only occurred after sufficient viral levels were achieved. In addition we should consider the potential for other arboviruses causing similar lesions in alligators. Further work is required to elucidate this theory.

CHAPTER 4: LYMPHOHISTIOCYTIC PROLIFERATIVE SYNDROME OF ALLIGATORS: A CUTANEOUS MANIFESTATION OF WEST NILE VIRUS

Introduction

Lymphohistiocytic proliferative syndrome of alligators (LPSA) and West Nile virus (WNV) have been previously described as diseases affecting captive reared American alligators (*Alligator mississippiensis*) (Nevarez et al. 2003, 2005b, Jacobson 2005a). Of these two diseases, WNV presents a public health concern. It has been shown that alligators can amplify WNV and serve as a reservoir for the virus (Klenk 2004). It is also known that personnel working in alligator farms at the time of a WNV outbreak have been exposed to the disease, and there is one confirmed case of direct exposure to WNV by handling tissues from an infected alligator (Tengelsen 2004). Until recently the etiology of LPSA was unknown. Past studies have ruled out a bacterial and fungal etiology, but were unable to conclude whether the disease was of viral origin (Nevarez et al. 2003b). Herpes viruses were initially suspected based on the histopathologic findings; however, polymerase chain reaction assays (PCR) were inconclusive (Nevarez 2005a). An association between exposure to WNV and the development of LPSA was described by Nevarez et al. (2006) following reports from Louisiana alligator ranchers about a possible link between WNV and the appearance of LPSA lesions in alligators that had survived WNV outbreaks.

The most challenging aspect of studying LPSA is the difficulty in correctly identifying the skin lesions ante-mortem. There are a number of gross lesions on the skin of alligators that are routinely misidentified as LPSA lesions. Despite this challenge, one of the authors (JGN) has extensive experience in identifying LPSA lesions based on gross observations combined with histopathologic identification. In addition, it had also become clear that LPSA animals would only be found in ranches with prior WNV outbreaks. Alligator ranches with a history of WNV would also have a history of LPSA and vice versa.

The objective of this study was to characterize the association of WNV with LPSA lesions in captive reared alligators. The specific hypotheses for this study were that animals with LPSA lesions would be significantly more likely to have WNV exposure based on antibody testing and WNV positive lesions based on RT-PCR tests.

Materials and Methods

This study was approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC). A total of 81, 6 to 9 month old, captive hatched and reared American alligators were used for the study. All alligators were obtained from a private rancher in Louisiana between March and June of 2006. The facility had a previous history of WNV and LPSA. The most recent WNV outbreak was diagnosed in October of 2005. Alligators were housed in rectangular buildings with appropriate stocking densities, water temperatures between 29 – 32°C (85°-90°F), and fed a commercial alligator diet that was occasionally mixed with whole ground chicken. Husbandry practices at the facility were comparable to other facilities in the state. One building contained animals with LPSA lesions, while another contained animals without LPSA lesions. Within each building alligators were selected at random. Animals were selected based on the presence (treatment group) or absence (control group) of LPSA skin lesions as identified grossly by one of the authors (JGN). The forty alligators selected for the treatment group (LPSA positive) originated from the only building in the facility in which LPSA had been observed. This also happened to be the same, and only building where, the WNV outbreak occurred in October of 2005. The additional 41 alligators representing the control group (LPSA negative) were obtained from a separate building in which no WNV or LPSA had been reported or diagnosed. All alligators from the treatment and control groups were collected and processed on different occasions to minimize the chance of cross-contamination. Alligators

were euthanized with Beuthanasia-D Special (Schering Plough Animal Health, Union NJ 07083) at a dose of 1ml/2.5kg administered in the supravertebral sinus.

WNV Antibody Testing

Each alligator was bled from either the supravertebral sinus or the lateral occipital sinus. Blood was placed in blood tubes with no preservative. The tubes were centrifuged at 5,000 RPM for 5 minutes before removing the serum and placing it in sterile vials. The serum was then stored at -70°C until being shipped with ice packs to the University of Florida for WNV antibody testing. The WNV antibody tests were performed using an indirect enzyme-linked immunosorbent assay for detection of antibodies to WNV in American alligators as reported by Jacobson et al. (Jacobson et al. 2005b).

WNV RT-PCR

A real time reverse transcriptase polymerase chain reaction test (RT-PCR) was performed on the skin and a liver/brain tissue pool from all animals. A 4mm punch biopsy was used to obtain the skin samples after determination that this biopsy size provided the amount of tissue needed for testing. An appropriate amount of liver and brain tissue was also obtained for WNV RT-PCR testing. In the treatment group, two skin biopsies were obtained. One biopsy was obtained directly over a scale with an LPSA lesion (TxA) in order to ensure that the lesions itself was being tested. A second skin biopsy was obtained from a scale section over which there was no LPSA skin lesion (TxB) present. This second biopsy was obtained as an additional intra-biopsy control. One potential pitfall of collecting the second skin sample in the treatment animals was that an LPSA lesion that was not grossly visible could have been obtained with the biopsy. In the control group a skin biopsy (CxS) was obtained from a scale after corroboration that there were no LPSA lesions in the whole ventral skin surface. Multiple biopsies were obtained from each animal in order to have additional tissues for banking. Brain and liver

samples were collected from each group during necropsy. All tissue samples (skin, liver/brain) were placed in a sterile plastic vial containing RNAlater[®] (Ambion Inc., 2130 Woodward St. Austin, TX 78744-1832 USA) at a 5: 1 ratio of solution to tissue and stored at -20°C until testing was performed. The RT-PCR procedure was performed according to a previously published protocol (Nevarez et al. 2005b).

Pathology

A gross examination was performed on each animal to document the presence or absence of LPSA skin lesions. Following euthanasia, a complete necropsy was performed on each alligator and tissues samples were collected and saved in 10% neutral buffered formalin for histopathologic examination. Preserved tissues were embedded in paraffin, sectioned to 5 micrometer thickness, adhered to a glass slide, and stained with hematoxylin and eosin using standard histologic procedures. Additional 5 micrometer sections of paraffin embedded tissues were tested for the presence of WNV using standard immunohistochemical staining.

Statistical Methods

The 95% binomial confidence intervals were calculated for each proportion. For these cases where the prevalence was 0, the technique described by van Belle was used (van Belle 2002). A Fisher's exact test was used to compare the treatment group and control group for each diagnostic (RT-PCR, serology, and histopathology). A $p \leq 0.05$ was considered statistically significant.

Results

WNV Antibody Testing

All 40 animals in the treatment group tested seropositive for WNV antibodies, while all 41 animals in the control group were seronegative for WNV. These results are consistent with a previous study that revealed an association between WNV seropositivity and the presence of

LPSA lesions (Nevarez et al. 2006). There was a significant difference ($p= 0.01^{-21}$) in the serostatus of the treatment group (100%) when compared to the control group between these results for the treatment and control group (0%, 95% CI: 0-7.3%).

WNV RT-PCR

Results for the RT-PCR are presented in table 16. In the treatment group, 97.5% (39/40) (95%CI: 92.7-102.3 %) of the LPSA skin lesions (TxA) were positive for WNV via RT-PCR. Of the skins within the treatment group that had no LPSA lesions (TxB), 7.5% (3/40) (95%CI: 0-15.7%) were positive for WNV. In the control group, all (40/40) of the skin samples (CxS) were negative for WNV (41/41) (0%, 95% CI: 0-7.3%). All alligators in TxA were significantly ($p=0.07^{-20}$) more likely to have RT-PCR WNV positive skin than those in CxS, and TxB ($p=0.08^{-16}$). There was no significant difference in the recovery of WNV from the skins of alligators from TxB and CxS ($p=0.24$).

For the liver and brain pool samples in the treatment group (Tx L/B), 67.5% (27/40) (95%CI: 53-82%) were positive for WNV via RT-PCR. In the control group all (41/41) (0%, 95% CI: 0-7.3%) of the liver and brain pool samples (Cx L/B) were negative for WNV. There was a significant difference between these results ($p=0.01^{-9}$).

Histopathology

Affected alligators had similar histopathologic lesions in the skin and superficial dermis. The superficial dermis, immediately beneath the epidermis, contained multiple round to ovoid

Table 16. Results of RT-PCR from skin and liver/brain pool from alligators. N=40

| Results | TxA ¹ | TxB ² | CxS ³ | Tx L/B ⁴ | Cx L/B ⁵ |
|----------|------------------|------------------|------------------|---------------------|---------------------|
| Positive | 39 | 3 | 0 | 27 | 0 |
| Negative | 1 | 37 | 40 | 13 | 40 |

¹TxA = skin from treatment group with LPSA lesion, ²TxB = skin from treatment group without LPSA lesion, ³CxS = skin from control group, no LPSA lesion ⁴Tx L/B = liver/brain pool from treatment group, ⁵Cx L/B = liver/brain pool from control group

lesions composed of large numbers of lymphocytes and macrophages (Figure 25). These foci were relatively sharply demarcated, contained variable numbers of tangible body macrophages, and sometimes surrounded small blood vessels. Within these accumulations of inflammatory cells, there was a marked loss of the thick collagenous stroma that makes up the normal dermis. There was a mild compression of collagen surrounding the lesions. The collagen extended a short way into the lesions and then ended abruptly. Small, scattered areas of collagenolysis were

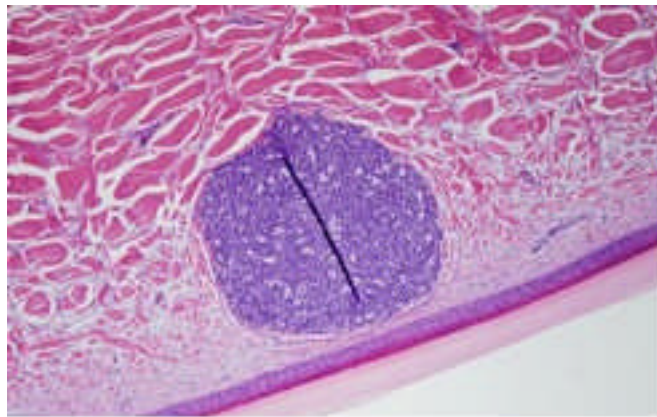


Figure 25. Typical LPSA lesion as observed under the microscope (40X).

noted around the margins of some of the lesions. The overlying epidermis was usually intact, but was sometimes mildly attenuated. Occasionally, lymphocytes extended into the epidermis immediately overlying the lesions. In these areas, the overlying scale occasionally contained foci of disorganized keratin above the epidermal lymphocytic infiltration. No organisms were seen within the skin or the superficial dermal lymphoid accumulations.

Discussion

The results of this study suggest a strong association between the presence of LPSA and WNV. To obtain definitive confirmation that WNV is responsible for the lesions, an experimental study fulfilling Koch's postulates will be required. Unfortunately, conducting such a study at this time is difficult due to the housing and biosafety requirements needed. To conduct such a study would require a BL-3 laboratory. Because of the large size and demands of these

animals, this is not possible at this time; however, plans to perform such a study are in progress. Nonetheless, without the privilege of having performed an experimental infection, the data clearly shows a very strong association and strongly suggest that WNV is highly correlated to the presence of LPSA lesions.

A disadvantage of the RT-PCR procedure is its inability to differentiate between viable (live) and dead virus. We attempted to culture WNV from five of the skin lesions but were unable to do so. This may have been explained by the chronicity of the disease leading to a lower amount of virus present in the tissue or the virus not being viable any more. One interesting finding that helps provide some insight into this matter is the fact that only 67.5% of the liver/brain pools tested positive in the treatment group as compared to 97.5% of the skin samples. This finding is consistent with an expected reduction in tissue levels with chronicity of the disease. Perhaps earlier sampling after the initial WNV outbreak would yield different results, having a higher proportion of tissues test positive for WNV and being able to culture the virus from the skin. The few positive results in TxB may be explained by the fact that LPSA lesions are sometimes difficult to visualize and in a positive skin, any given section has the possibility of housing a lesion.

Skin manifestations associated with WNV have been described in humans, although histopathologic evaluation of the lesions has been limited. Nonetheless, there are reports of exanthema and rash lesions associated with WNV infection dating back to 1956 (Marberg et al. 1956, Cernescu et al. 2000, Asnis et al. 2000, Nash et al. 2001, Klein et al. 2002, Estival et al. 2001, Seivar et al. 2003, Pealer et al. 2002, Ferguson et al. 2005, Anderson et al. 2004). There is also a report of WNV identification in the skin of two Goshawks (*Accipiter gentilis*) via immunohistochemistry (Wünschmann et al. 2005). These reports support our findings of LPSA as a skin manifestation of WNV. We believe this type of inflammation in the skin of alligators

may be prompted by an aggressive immune response secondary to chronic exposure to WNV but further studies are required to understand the immune response to the virus.

CHAPTER 5: PREVENTION, SURVEILLANCE, AND CONTROL METHODS FOR WNV IN ALLIGATOR RANCHES

Introduction

It appears that WNV will remain an important enzootic disease in the United States, and that we should continue to focus on designing effective surveillance, prevention and control methods that will decrease morbidity and mortality in both human and animal populations. For alligator ranches, this goal is no different in light of a lack of effective treatment for animals and humans infected with WNV. The economic impact of WNV in alligator ranches in Louisiana between 2003 and 2006 was estimated at over 8 million dollars, with a total loss of over 50,000 alligators. This represented losses from dead alligators only, and does not take into account those that survived WNV infection but later developed LPSA. The true economic impact of LPSA is currently unknown. Because these financial losses seriously impacted the Louisiana alligator industry, local ranchers mobilized to seriously consider and implement prevention and control plans for WNV in their facilities. The key to decreasing and possibly eliminating WNV outbreaks in alligator ranches lays in the effective implementation of surveillance, prevention, and control methods. The three are closely associated, and together should form part of an overall program for WNV control in alligator ranches.

Prevention

The first step in designing a prevention program is to understand the biology and ecology of the disease in question. One must be able to identify the host and vector relationships, as well as the environmental conditions that stimulate or inhibit the spread of the disease. In the case of WNV, its life cycle primarily consists of birds (mostly passeriformes) as reservoir hosts and mosquitoes as primary transmission vectors. Humans and animals are incidental hosts, some with the ability to amplify the virus (e.g., alligators) and others being dead end hosts (e.g., horses and humans). Of the two key players in the cycle of WNV, birds and mosquitoes, prevention

methods can be more easily applied to the transmission vector. For any control plan to be effective, it is important to break the life cycle of the organism. One major challenge in the control of WNV is the improbability of eliminating all the mosquitoes within a specific geographic area. In addition, mosquito control does not eliminate the reservoir host. Once these challenges are recognized, it becomes important to effectively spend the money and resources available for controlling the disease.

The mainstay of WNV prevention is eliminating, or at least decreasing the number of vectors. This can be done through aggressive mosquito control, with the intent to minimize the amount of mosquitoes that can potentially carry the virus and infect alligators. Both non-chemical and chemical mosquito control methods can be used. The control methods are aimed at reducing both larval densities, as well as the adults. The most basic element of mosquito control involves the elimination of habitats that promote breeding. Emptying containers that can hold stagnant water (e.g., tires, feed troughs, plastic bins), keeping the grass cut, agitating the water in ponds, and even painting the buildings in light colors that are less attractant to mosquitoes, are all non-chemical methods that can be used to help control mosquitoes in an alligator facility. In addition, it is also important to minimize the likelihood of mosquito entry into alligator buildings. Buildings should be inspected for cracks or other damage that allows mosquito entry, and any building defects should be repaired immediately. One major entry way for mosquitoes into alligator buildings is via gaps in the doors or during times of feeding and cleaning when the doors remain open. These problems can be alleviated by adding appropriate weather stripping to the doors to ensure a tight seal when closed. Screen doors can also be added to the doorways so that while feeding and cleaning, the main doors can remain open but the screen doors prevent mosquitoes from entering the building.

Unfortunately, non-chemical methods alone are insufficient to control mosquito populations. Active spraying for mosquitoes and treating against larvae is a critical aspect of the prevention strategy. These treatments may be performed by local agencies or by the owners of the facilities themselves. While some mosquito control agencies have provided guidance and assistance to alligator ranchers, ultimately the owners of the facility have to take on the primary responsibility of spraying chemicals to decrease mosquito populations. Many ranch owners have purchased the necessary equipment and chemicals, but should consult with mosquito control agents or the companies that manufacture the products to ensure their success. Type of application, frequency, time of day, and environmental conditions are some of the factors to consider when using these chemicals to ensure their effectiveness as well as limit their impact on the environment.

An additional prevention strategy in alligator ranches deals with the proper disposal of carcasses from animals that died of WNV infection. Because alligators can serve as an amplifying host, proper disposal of infected carcasses is essential. Current recommendations are to immediately burn carcasses or freeze them until the time of burning. In the past, most ranchers disposed of their carcasses above ground in fields within close proximity to their facility, creating a possible source of live virus for vectors. Fomites also represent a source of disease transfer between alligator buildings. The use of foot baths and hand washing stations outside every building would minimize the potential of disease transfers within a facility. Each building should also have a separate set of tools such as feeding buckets, nets, and any other object that are routinely used within the building. Ideally each building would also have a designated pair of rubber boots for each worker. These tools should also be disinfected after each use. A critical part of these preventive methods is to ensure that the disinfectants being used (e.g., bleach, clorhexidine) are changed daily in order to maintain their effectiveness.

Since the initial WNV outbreaks in alligator ranches during 2003, there has been an increase in the mosquito control efforts at various alligator facilities in the state of Louisiana. The owners of the facilities have invested in equipment to spray for mosquitoes, altered their buildings and even altered management techniques to prevent WNV infection in their facility. Improved weather stripping of doors has been observed in some facilities in an attempt to prevent mosquito entry. Others have added screen doors so that while the main doors remain open during cleaning, the screen doors prevent mosquito entry. One facility has implemented an automatic feeding system that minimizes entry into the buildings and consequently decreases the likelihood of mosquito entry. More alligator ranchers are also conscious about the risks of stagnant water and perform routine inspections of their facilities to empty any containers holding water. The long term effects of these programs will continue to be assessed in the future.

Surveillance

The term surveillance has been assigned multiple definitions by different authors over the years, and there is no stand alone definition that is universally accepted (Thrusfield 2005). The types of surveillance are also varied, and range from sentinel surveillance to serologic surveillance and active and passive surveillance (Thrusfield 2005, Moore et al. 1993). Perhaps the most important distinction between the types of surveillance is whether it is passive or active. Passive surveillance relies on examination of clinically affected animals only and therefore occurs once the disease is present within the population. Active surveillance involves the examination of clinically normal animals, and therefore has the advantage of being able to detect the presence of subclinical and/or carrier animals within the population (Thrusfield 2005). Ideally, a disease surveillance program would incorporate both passive and active methods of surveillance. The type of disease, diagnostic tests available, funding, and personnel are some of the factors that influence which method is employed. For our purposes, we will define WNV

surveillance as the organized gathering and analysis of virus activity as it pertains to its presence in vectors, as well as exposure and infection in animal hosts, in order to appropriately allocate and implement available resources for control and prevention of disease. The ultimate goal of surveillance programs in alligator ranches would be early identification of virus activity within a facility before alligators are exposed or infected. This in turn would lead to rapid and continued implementation and follow up of vector control and other prevention and control methods. Surveillance would also allow for evaluation of current methods of controlling vectors and determining the effectiveness of those methods. Implementation of an active surveillance method to parallel applied passive surveillance methods would provide the best approach to control WNV in alligator facilities.

WNV surveillance can be performed using sentinel birds and/or trapping mosquitoes. Sentinel birds should be placed in a location where WNV is suspected to be a problem based on past experiences or models that predict that an area is a suitable habitat for reservoir and vector. The ideal sentinel bird should be susceptible to infection, resistant to disease, and mount a rapid immune response that can be detected with available antibody tests such as an ELISA (Komar 2001). It is also important for these birds to undergo seroconversion before the onset of the disease outbreak within the geographical location. Finally, these birds should be easy to maintain, present minimal health risks to the handlers, and must not contribute to the transmission cycle of the virus (Komar 2001). The sentinel birds are tested for the presence of antibodies to WNV on a regular interval (weekly or biweekly). If antibodies are detected at any time, it would be an indication that WNV is present in the area and prevention methods should be heightened.

One major limitation of using sentinel birds, such as chickens, is a historical lack of success with these programs around the nation (Komar 2001). It is reported that in many states,

the positive sentinel chickens were identified after the onset of human cases (Komar 2001). Some possible explanations for the limited success of these programs may lay in the design of the sentinel program itself. The cage design and location, bird species, height above ground, flock size, and proximity to susceptible populations are all factors that will influence the success of sentinel programs (Komar 2001). In alligator ranches, there is the advantage of having a well defined geographical area defined by the boundaries of the property. Once these boundaries are defined, further breakdown of possible sites for locating sentinel chickens can be defined based on the known location of mosquito larvae as well as adult mosquitoes. Because of limited land space in most facilities, these areas of larvae and adult mosquito concentration also happens to be in close proximity to the susceptible population of alligators. Together these two elements facilitate the placement of the sentinel birds and increase the chances of success of the program. One major limitation would be the attractiveness of the sentinel birds versus the alligators themselves. There can be between two and five thousand alligators within a building, which may prove to be more attractive for mosquitoes than a few sentinel birds in a cage. The inside of the alligator buildings will also have a higher temperature, humidity, CO₂, and organic odor than the outside environment. These factors may greatly hinder the effectiveness of sentinel programs in alligator facilities because it will simply be too difficult to compete against the environment within an alligator building when it comes to attracting mosquitoes.

An alternative to captive sentinel birds is utilizing free ranging birds within the property. Free ranging birds will cover more ground and would not be limited to a single location. Sentinel free range birds could include domesticated birds within the property as well as wild birds captured and bled for purposes of testing. The capture of resident wild bird species would require a permit, while non-native species, such as the house sparrow (*Passer domesticus*), European starling (*Sturnus vulgaris*), and the domestic pigeon (*Columba livia*), can be captured

and tested without a permit. The use of free ranging sentinels also has its limitations as they would have the ability to move into areas with low mosquito concentrations and would also be competing with the alligators for attracting mosquitoes. There is also a degree of difficulty in trapping the birds, and determining when exposure to WNV occurred. From this information it is evident that sentinel programs have certain limitations that should be weighed against their cost and effectiveness. Perhaps it is important that the alligator facilities be evaluated further by personnel familiar with sentinel programs, such as entomologists, in order to determine the potential success of these programs. One major issue that must be addressed is the attractiveness of the alligator buildings themselves when compared to other hosts in the property. Ultimately sentinel surveillance is aimed at providing information about the risk level in an enzootic region.

A second surveillance system that can be employed is vector (mosquito) surveillance. This can be further divided into larval and adult surveillance. Identification of potential breeding grounds and larval habitat is critical for estimating future vector populations as well as treating the larva (Moore et al. 1993). Surveillance of adult mosquito populations will provide information about species, density, seasonality, viral infection, and minimum infection ratios (MIR) (White 2001). In addition, adult vector surveillance can be used to monitor the effectiveness of prevention programs. While larval surveillance is important, adult mosquito surveillance is critical in determining the presence of WNV in the vector within a geographical region. Various methods are available for trapping mosquitoes for surveillance purposes. The alligator industry should seek help from individuals trained in mosquito surveillance to design appropriate plans for their facilities. The owners of the alligator facilities could also have trained personnel within their operations to set the traps and collect the mosquitoes, which then could be shipped for WNV testing. This would limit their dependency on local agencies. Mosquito surveillance plans also have some basic requirements for their success. Utilizing the appropriate

equipment and supplies, time and placement of the traps, species identification, and appropriate handling of samples are all critical for the success of a mosquito surveillance plan. In addition, there must be effective and diligent communication between the laboratory testing the mosquito pools for WNV and the alligator ranchers/farmers so that the information obtained can be directly applied to the prevention and surveillance methods.

Control

Despite the implementation of prevention and surveillance practices, WNV may still make its way into an alligator building and infect the animals within. In these instances, it is important to have a control plan in place to prevent further spread of the virus and decrease the morbidity and mortality levels. The first and most essential step is strict isolation of any building(s) housing the infected/exposed animals. This strict isolation of a building would restrict any movements of animals in and out of that building, and so far has proven to be an effective way of preventing the spread of WNV within a facility. Part of this strict quarantine includes enforcing the use of foot baths and hand washing stations, as well as the disinfection of tools that must only be used in the affected building. Feeding and cleaning must be performed last in the affected building, as an added measure to minimize the spread of disease. Ideally there would only be one individual designated to work in the affected building, and/or it would be required that any persons working in that building take a shower and change clothes immediately after their work is completed. Due to the zoonotic nature of WNV, the individual working in the affected building must not be immunocompromised. While not common, human infection amongst workers during a WNV outbreak in an alligator facility has occurred. Some of these prevention methods have already been successfully implemented during some of the WNV outbreaks in Louisiana.

Other methods of control have been explored, but their effectiveness has yet to be determined. One promising technique is the active control of water temperatures in alligator buildings according to time of the year and WNV exposure. While little is known about the pathogenesis of WNV, temperature is believed to play a role in viral replication. Temperature associations have been documented for the replication of WNV in *Culex pipiens* (Dohm and Turell 2001). Dohm and Turell experimentally infected mosquitoes by allowing them to feed on chickens inoculated with 10^4 pfu/ml of WNV (Dohm and Turell 2001). This study showed that engorged mosquitoes held at 26°C (79°F) had detectable virus while engorged mosquitoes held at 18°C (65°F) and 10°C (50°F) had no detectable virus (Dohm and Turell 2001). Furthermore, if the mosquitoes held at 18°C (65°F) and 10°C (50°F) were then kept at 26°C (79°F) for as little as 1 day, WNV was detected (Dohm and Turell 2001). Mosquitoes held at 26°C (79°F) had 100% infection rate with increased time of incubation (Dohm and Turell 2001). A similar temperature association has been observed for infection of WNV in alligators. The author has observed three instances of a sudden decrease in the identification of WNV via RT-PCR from liver and brain of captive reared alligators during a WNV outbreak. In all instances, the sudden decrease in the identification of WNV occurred after the water temperatures in the alligator buildings dropped below 26.6°C (80°F) due to a failure in the water heaters or artesian well pump. This clinical evidence adds support to the possibility of there being an internal body temperature threshold that is necessary for WNV to replicate in a host's cells. It is also interesting to note the differences observed in the pathogenicity of WNV between captive reared and free-ranging populations of alligators. In a study by Jacobson et al., only 1.5% (10/669) of free-ranging alligators tested positive for WNV antibodies (Jacobson et al. 2005). There is also no indication that WNV is responsible for mortalities of free-ranging alligators. These data differ from what is usually observed in captive reared alligators in which mortalities from WNV

can reach up to 60% (Nevarez et al. 2005). Captive reared alligators are maintained at constant water temperatures between 29°C (85°F) and 32°C (90°F), which in turn allows the alligators to maintain body temperatures closer to what may be expected in mammals. In contrast, free-ranging alligators in a natural environment are subjected to a circadian fluctuation in environmental temperatures during a 24 hour period. In the wild, the temperature fluctuations could possibly vary as much as 15 – 20 degrees. These environmental fluctuations will also lead to internal body temperature fluctuations in free-ranging alligators. This is especially true at night when free-ranging alligators may have lower body temperatures that would lead to a hypothermic state in mammals.

These differences between captive and free ranging alligators reveal the possibility of a strong temperature effect on the susceptibility of captive reared alligators to WNV infection. While it could be argued that stocking densities may also contribute to increased number of exposure within captive populations, there are many areas in Louisiana that have high concentrations of free-ranging alligators and yet fail to show any impact from WNV infection based on a lack of increased mortality reported from these areas. It is also interesting to know that no other species of reptiles has been show to be affected by WNV to the extent that captive reared alligators are affected. Once again, a major difference in the husbandry of other reptile species raised in commercial operations is that alligators are constantly housed at warm water temperatures, while these other reptiles are not. This begs the question of whether preemptive control of water temperatures by allowing circadian fluctuations in a captive environment, similar to a natural environment, may decrease the occurrence of WNV infection in captivity.

While there is much left to learn about this association, there are some possible applications that could be pursued in the meantime. One possibility would be to determine the seasonality of WNV, and the most likely time of the year for infection in captive alligators. With

that information, the alligator producers could then change the water temperature scheme to match a more natural variation during the months of the year with a higher risk of WNV infection. For example, between the months of August to December, when most WNV outbreaks in alligators occur, water temperatures within an alligator building could be made to fluctuate between 24°C (75°F) and 29°C (85°F) in a 24 hour period. If the temperature association is true, one might expect to see a drastic decrease in the occurrence of WNV in alligator ranches. The trade off of these temperature fluctuations would be a slower growth rate, which may lead to an extended period in captivity before reaching market size. An alternative would be to rely on surveillance and prevention methods and only decrease the water temperatures if a WNV outbreak occurs. At this point, the specific temperature and period of time for which this temperature change should be maintained are unknown. However, based on the clinical cases previously mentioned, a drop in water temperature to 24°C (75°F) for 14 days may drastically reduce WNV infection within a captive population. The state of these alligators once the temperatures are raised back again is unknown. One major question is whether susceptibility would increase again and whether these animals would still be capable of shedding the virus.

An additional control method may rely on reducing the amount of WNV exposure by treating the water. It is known that alligators can amplify WNV and shed it in the feces. Fecal oral contamination is probably the primary route of WNV spread within a building after initial exposure of some animals via a mosquito. At this point it is unknown whether the virus can survive in the water, for how long, and at what levels. In the future, the use of disinfectants (e.g., bleach, clorhexidine, Baquacil (Arch Chemicals Inc., Norwalk Connecticut)) in the water may be an important part of the control strategies for WNV.

Antibiotic therapy may also be warranted in some case when secondary bacterial infections occur in animals infected/exposed to WNV. If alligators infected with WNV are also determined to have a secondary bacterial infection, such as pneumonia, a culture and sensitivity should be obtained during necropsy in order to identify the offending bacteria and ensure that the selected antimicrobial is appropriate for the infection. Antimicrobial treatment of remaining animals may improve their chances of survival.

Discussion

The available information about WNV infection in captive alligators has increased over the past 5 years, but there are still many unanswered questions. What is clear is the fact that captive alligators are susceptible to WNV, and that there must be a plan in place to minimize exposure. This plan must rely on surveillance for WNV in the environment, preventive methods to minimize exposure, and control methods to decrease the spread of the virus if infection occurs. The integration of all three areas is necessary in order to be successful in decreasing morbidity and mortality. It is also important to recognize that WNV outbreaks in alligator facilities have both an acute and a chronic effect leading to monetary losses. This makes WNV an even more devastating disease for the alligator producers. It now appears that once WNV occurs in a facility, LPSA will also occur. With this in mind we must realize that primary efforts should concentrate on the surveillance and prevention of WNV infection. Once an outbreak occurs the goal is to minimize losses realizing that an economic impact will be felt.

The alligator industry must look beyond their operations and continue to recruit experienced individuals from the veterinary, entomology, ornithology, and virology fields that can help them design cost effective plans for the surveillance and prevention of WNV. Management changes are also essential in these facilities. Some have already started implementing changes, such as maintaining tightly sealed buildings, adding screen doors, and

utilizing foot baths. These methods must become part of the daily management of these facilities and not allowed to become lax. We are hopeful that these measures will lead to a decrease in the amount of WNV cases in the future.

CONCLUSIONS

After three years of investigation, we have gathered sufficient evidence to suggest that the presence of LPSA is significantly associated with the serologic and molecular manifestations of WNV infection in captive reared alligators. Furthermore, we have been able to characterize two disease stages of WNV in alligators, an acute and a chronic stage. The acute stage of WNV in alligators has been previously characterized by a primary heterophilic inflammatory response in the tissues (Nevarez et al. 2005b), while the chronic stage of WNV is characterized by the presence of lymphohistiocytic infiltrates. Gross examination, histopathology, RT-PCR, and WNV serology have all been used to provide a link between the presence of LPSA and WNV. The most striking results are presented in chapter four, which revealed a statistically significant difference between TxA (skin from treatment group with LPSA lesion) and both CxS (skin from control group, no LPSA lesion) and TxB (skin from treatment group without LPSA lesion) (Table 16) that indicates that WNV is present within the LPSA lesions themselves. A similar skin manifestation associated with WNV infection has also been reported in humans and birds (Anderson et al. 2004, Wünschmann et al.2005). The results of this study have shed new light into the epidemiology of WNV in a species of reptile, and also revealed that the pathologic responses observed in these animals are similar to those described for other vertebrates (e.g., birds, mammals).

It is interesting that this disease appears to be primarily associated with captive alligators. To date, there has been a single study evaluating WNV exposure in alligators from the wild (Jacobson et al. 2005b), and the prevalence of disease appears to be low. On the surface, the disease noted in the captive animals may appear to be associated with the high stocking densities of animals; however, the physiologic conditions of the captive animals are also different, and should be considered. Captive alligators are maintained at temperatures that are constant (29.°C-

32.°C; 85°F – 90°F), allowing them to maintain body temperatures that are more similar to mammals. The shift to a warmer body temperature may be sufficient to allow the virus to replicate in the alligators. In contrast, wild alligators are exposed to circadian changes in temperature, and may experience body temperatures well below that which the virus can replicate at. There remains more to be learned about the immune response of alligators to infectious agents. Cellular immunity in such a primitive species is likely to play a more significant role than what is currently recognized. While the humoral response will also have a significant contribution, it is unclear how both of these responses work together in alligators. One theory in this respect is that the cellular response has the primary domain in alligators and that LPSA lesions are the result of a severe response to an antigen, in this case WNV. This cellular response to the chronic presence of an antigen is so drastic that it eventually leads to focal obliteration of normal tissue (e.g., connective tissue in the dermis) in order to eliminate the offending antigen. Further studies are required to better understand the immune response in alligators and its effect on different types of antigens.

As we learn more about the epidemiology of WNV, it has become more apparent that the life cycle may incorporate more than just birds and mosquitoes. As it has been shown, alligators have the potential of playing an important role in the cycle of WNV (Nevarez et al. 2005b) (Figure 26). Based on this research, the epidemiology of LPSA appears to mimic the epidemiology of WNV. The virus is maintained in the environment by birds serving as reservoir hosts. Mosquitoes will bite these birds and serve as a vector for the virus. Mosquitoes living in the alligator ranches are attracted to the alligator buildings housing thousands of animals exhaling CO₂ and producing a large amount of organic waste that creates a warm, humid environment even during the coldest time of the year. Mosquitoes will bite alligators on the oral mucous membranes, between the scales, or around the

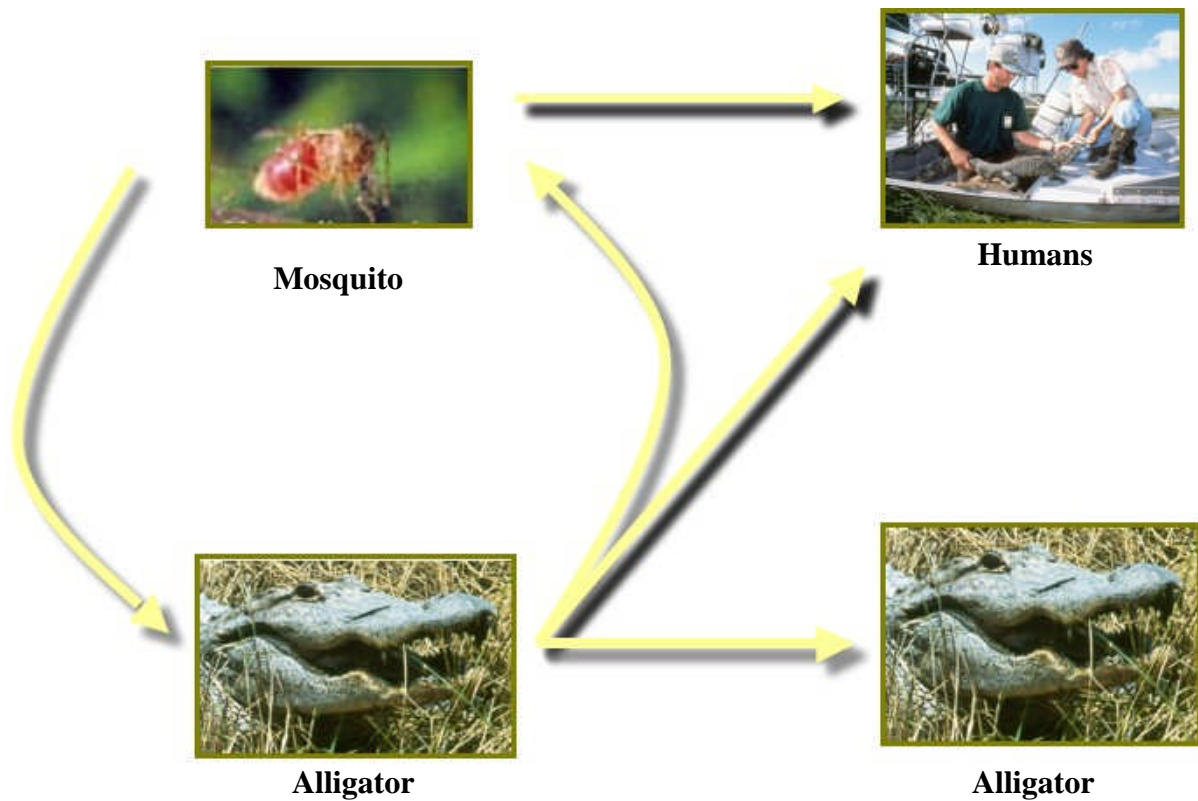


Figure 26. Role of Alligators in the cycle of WNV

eyes, where the skin is softer and easier to penetrate. Once infected, the internal body temperature of the captive alligators provides an appropriate environment within its cells for WNV to replicate and be amplified. The virus is then shed via the feces. High viremias are also likely maintained for an unknown period of time. Viral spread will be possible via fecal oral contamination and/or by mosquitoes feeding within the buildings. This will lead to a rapid spread of the virus within a building that can reach mortalities of 60% (Nevarez et al. 2005b). Alligators that survive the WNV outbreak will continue to thrive, but frequently develop skin lesions within 4 weeks of exposure to WNV. These skin lesions appear to be the manifestation of a chronic WNV infection/exposure.

The findings of this study raise important questions regarding the epidemiology of WNV in the United States. “PIX” disease was first described in Florida in 1999, and is histologically

similar to LPSA (Cardeilhac et al. 2001, Nevarez et al. 2003a, Nevarez et al. 2003b). If LPSA and PIX disease represent the same disease process, then WNV was potentially circulating in Florida in 1999; the same time it was first diagnosed in New York City, New York (CDC 1999). This would suggest that WNV was not introduced into the United States in 1999, but at least one year earlier. Based on our general knowledge of the epidemiology of other arboviruses, this would not be unexpected as disease is not generally diagnosed until there is sufficient viral spillover to humans or domestic species. It might be expected that WNV could be disseminated between New York City and Florida via bird migration. Based on these assumptions, it is possible that WNV was first introduced into Florida, and that the 1999 outbreak in New York only occurred after sufficient viral levels were achieved. At this time we have been unable to obtain “PIX” affected tissues from alligators (1999 samples) in order to perform WNV testing and determine if those lesions were indeed the similar to the Louisiana LPSA lesions. It is interesting to note that of the three cases of WNV in Louisiana alligator farms in 2003, two occurred after importation of Florida alligators infected with WNV. The United States Department of Agriculture, Wildlife and Fisheries, and veterinarians alike should work together to develop requirements for the import/export of alligators that include surveillance of WNV in alligator facilities. West Nile virus is just one example of how diseases may be crossing state lines and ultimately creating the potential for devastating effects both in the captive and wild populations of alligators. This surveillance would be important even between WNV endemic states, since we can not ensure that alligator producers in other states are implementing the same WNV prevention methods as in Louisiana. If alligators continue to be imported from Florida, WNV may still make its way into the state despite the best surveillance, prevention and control programs.

Fortunately, the alligator production setting in Louisiana is primarily an all-in and all-out operation, which allows us to break the cycle of the virus and take preventive measures before introducing new animals. Surveillance, prevention, and control methods for WNV will continue to be an integral part of the daily management of alligator ranches across Louisiana and other alligator producing states. We will continue to monitor WNV activity in and around alligator facilities and observe the effectiveness of the recommendations for WNV surveillance, prevention and control in the years to come. If these recommendations are implemented, the incidence of WNV in alligator ranches should decrease drastically. As of the spring of 2007, there has been less concern from alligator ranchers about the occurrence of LPSA. In 2006, there was only one case of WNV in an alligator facility. This reveals what is likely the beginning of a decline in the occurrence of WNV and LPSA in alligator ranches. Once the effects of temperature on the cycle of WNV are elucidated, temperature changes in the buildings may also be used as a control tool for WNV.

This project has revealed an interesting connection between husbandry practices in alligator facilities and the occurrence of WNV. As mentioned previously, the temperatures under which these alligators are raised likely plays a key role in allowing WNV to adapt in these animals. This should create awareness in the reptile community about the fact that reptiles are just as susceptible to what were previously thought to be mammalian diseases, if maintained at the right environmental conditions. Finally, we must also consider the possibility that other arboviruses may be able to cause the same or similar tissue reactions in alligators. They would remain important because of their economic impact. While we did not test for any other arboviruses, diagnostics should be considered when similar lesions arise in the face of WNV negative tests. However, with the long history of eastern equine encephalitis and St. Louis

encephalitis in Louisiana, and the absence of clinical disease prior to the arrival of WNV, this point may be moot.

There are still future studies that need to be performed in order to completely define the relationship between the presence of LPSA lesions and WNV positivity. A WNV infectivity study would be ideal in order to meet Koch's postulates and obtain a definitive answer. On the other hand, the type of lesion observed with LPSA is a nonspecific type of lesions that is usually associated with a viral infection. Therefore, as mentioned previously, there is a possibility that this reaction may not be restricted to WNV alone but could also be elicited by other flaviviruses. An infectivity study would also allow us to follow the progression of LPSA and determine the time occurrence from the time of infection. It is also important to be able to monitor LPSA positive animals and determine if the lesions will egress at some point. So far we have no evidence to suggest that the lesions will heal or egress and the amount of pathology in the tissues make a complete healing unlikely.

In addition to the infectivity study, we need to examine more carefully the shedding and transmission cycles of the virus in alligator facilities. We believe that primary transmission occurs within an affected building via fecal-oral contamination, after the initial animals are infected via a mosquito bite. However, the amount of fecal shedding and viremia that occurs in these animals is unknown at this time. Knowledge of the survivability of WNV in water, especially water saturated with organic material, should also be pursued. If the virus was determined to survive in the water, then studies could be performed to determine the efficacy of various compounds (e.g. salt, chlorine, clorhexidine) at eliminating the virus from the water.

The pursuit of this information is required to better define the epidemiology of WNV within alligator facilities, and ensure that the surveillance, prevention, and control methods are adjusted accordingly. Even without this knowledge, it is becoming evident that our efforts and

recommendations have had a positive impact on the alligator industry and the occurrence of WNV and LPSA has begun to decline.

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**APPENDIX: LETTER OF PERMISSION FROM THE JOURNAL OF
HERPETOLOGICAL MEDICINE AND SURGERY**



March 8, 2007

Javier G Nevarez, DVM
LSU School of Veterinary Medicine
Skip Bertman Dr.
Baton Rouge, LA 70803

Dear Dr. Nevarez,

The Journal of Herpetological Medicine and Surgery (JHMS) grants you permission to include the manuscripts entitled "West Nile Virus in Alligators, *Alligator mississippiensis*, Ranches from Louisiana" and "Establishing an Association between West Nile Virus Seropositivity and Lymphohistiocytic Proliferative Syndrome of Alligators" as chapters in your dissertation to fulfill requirements of the PhD program.

Sincerely,

Lilia Boyer
Assistant Editor
JHMS
3454 Chasewood Drive
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858-541-2085

VITA

Javier G. Nevarez was born in San Juan, Puerto Rico, on September 20, 1976. He graduated from the high school El Colegio San Ignacio de Loyola in May of 1994. That same fall, Javier began undergraduate studies at the Louisiana State University in the animal sciences three plus one program. In 1997, Javier was admitted to the Louisiana State University School of Veterinary Medicine. Javier obtained his Bachelor of Science degree with a concentration in animal science in May of 1998, and his Doctor of Veterinary Medicine degree in May of 2001. Following graduation from veterinary school, Javier completed a one year internship in zoological medicine at the Louisiana State University School of Veterinary Medicine in June of 2002. After completion of the internship, Dr. Nevarez enrolled in a Doctor of Philosophy program at the Department of Veterinary Clinical Sciences in the Louisiana State University School of Veterinary Medicine. In 2003, Dr. Nevarez was hired as an instructor of zoological medicine in the Veterinary Clinical Sciences department. Since that time, Dr. Nevarez has continued to work towards the fulfillment of his Doctor of Philosophy degree requirements as well as carrying out teaching and research responsibilities within the Louisiana State University School of Veterinary Medicine.