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Detection of the bloodmeal origin of mosquitoes collected at alligator farms, and West Nile Virus activity in mosquitoes collected from alligator farms, sentinel chicken surveillance sites, and larval habitats

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DETECTION OF THE BLOODMEAL ORIGIN OF MOSQUITOES COLLECTED AT ALLIGATOR FARMS, AND WEST NILE VIRUS ACTIVITY IN MOSQUITOES COLLECTED FROM ALLIGATOR FARMS, SENTINEL CHICKEN SURVEILLANCE SITES, AND LARVAL HABITATS

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Dedicated to the late Dr. Michael Perich (the wind beneath my wings)

and my husband Emre Unlu

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ABSTRACT

A three year study was conducted to determine if testing mosquitoes collected in modified sentinel chicken boxes for West Nile Virus (WNV) or testing sentinel chickens for WNV antibody would detect WNV activity prior to reports of human cases in East Baton Rouge (EBR) Parish, Louisiana. In one year we also compared the effectiveness of CDC light traps, gravid traps, and sentinel chicken box traps for collecting WNV positive mosquitoes. In all three years, we detected WNV activity in mosquito collections from sentinel chicken box traps prior to the onset of human cases, while there were no seroconversions in the chickens prior to human cases. In order to incriminate mosquitoes as vectors of WNV for captive alligators, mosquitoes were collected using Centers for Disease Control (CDC) light traps, gravid traps, backpack aspirators and resting boxes at three commercial Louisiana alligator farms from 2004 to 2006. The bloodmeal origins of 237 field-collected mosquitoes were identified based on cytochrome B (*cytb*) sequence homology. Alligator blood was detected in 24 mosquitoes representing six species of mosquitoes. This is the first study that identified alligator blood from mosquitoes at Louisiana alligator farms. Mosquitoes also were collected from the commercial Louisiana alligator farms and tested for WNV RNA. A total of 2, 404 mosquito pools were tested using reverse transcriptase polymerase chain reaction. West Nile virus was detected in 41 pools of females of 11 mosquito species. A comprehensive survey for vertical transmission of WNV was conducted. Male mosquitoes and mosquito larvae were collected in EBR Parish, Louisiana, using CDC light traps, gravid traps, backpack aspirators, resting boxes, and from larval habitats. West Nile virus was detected in 15 pools of male *Culex* specimens (collected as adults). West Nile virus also was detected in

2 pools of *Cx. quinquefasciatus* nulliparous females, 1 pool of *Ae. albopictus* nulliparous females and 2 pools of *Culex* species males. This is the second report of WNV vertical transmission in nature for *Cx. quinquefasciatus* and the first study that detected WNV from field collected nulliparous *Ae. albopictus* females.

INTRODUCTION

West Nile virus was first identified in 1937 from blood of a febrile woman in the West Nile District of Uganda. The virus became known as West Nile Fever in North Africa and the Middle East during the 1950's, and was occasionally isolated from febrile children. In 1957, in an outbreak amongst elderly patients in Israel, the virus was recognized as a cause of serious central nervous system infections (Hayes 1989).

The introduction of WNV into the New World was first detected in the summer of 1999 in New York City, and public health authorities reported 59 hospitalized human cases and 7 deaths during 1999. The subsequent rapid spread of WNV within the continental United States during 1999-2006, indicates that WNV found efficient vectors, suitable vertebrate amplifying hosts, and reliable overwintering mechanisms in many different environments. Investigation of the life history of the WNV after its introductions into new ecosystems is important to understand the epidemiology of the virus.

West Nile virus activity was first recognized in East Baton Rouge (EBR) Parish, Louisiana in 2002, with the isolation of the virus from a dead Northern Cardinal collected in February and from a live male cardinal sampled in March. The Louisiana Department of Health and Hospitals has reported 915 WNV human cases including 60 deaths during the period of 2002-2007 in Louisiana. Since there is no WNV specific treatment or vaccine available, the prevention of human disease is strongly based on effective surveillance programs, sustained mosquito control, and public education. An objective of studies presented in this dissertation was to determine if testing mosquitoes or chickens from modified sentinel chicken boxes for WNV would provide information for early warning of WNV activity prior to human cases and provide information for timely intervention.

West Nile virus was first reported to infect American alligators (*Alligator mississippiensis*) in the United States in 2001, and more than 700 WNV-associated hatchling deaths occurred at three Louisiana alligator farms in 2003 (ProMed-mail, 2003). The second objective of this dissertation was to conduct studies on vector incrimination of mosquitoes as vectors of WNV for captive alligators. Recent studies have provided evidence of vertical transmission of WNV in mosquitoes. Miller at al. (2000) first reported vertical transmission for WNV in nature for *Cx. univittatus* (Theobald) males collected from Rift Valley Province of Kenya. The last objective of this dissertation was to screen for vertical transmission in EBR Parish in Louisiana from field collected adult male mosquitoes and mosquito larvae.

CHAPTER 1: LITERATURE REVIEW

Mosquitoes were formally incriminated as vectors of vertebrate parasites in 1878 (Woodbridge and Walker 2002). Today mosquitoes are recognized as the most important arthropods affecting human and animal health. Mosquitoes transmit the agents of such well-known human diseases as malaria, filariasis encephalitis, yellow fever, and dengue. Mosquitoes are estimated to transmit disease agents to more than 70 million people annually. The World Health Organization reports 3 million deaths annually from malaria alone (Fradin 1998).

Taxonomy of Mosquitoes

Mosquitoes are in the family Culicidae, which belongs to one of the largest orders of insects, the Diptera. Mosquitoes are distributed in every region of every continent except Antarctica. Their habitats include arctic tundra, boreal forests, high mountains, plains, deserts, tropical forests, salt marshes, and ocean tidal zones (Woodbridge and Walker 2002).

Culicidae consists of over 3500 recognized species and there are probably many more species remaining to be described (Goddard 2007). Culicid classification currently has three subfamilies: Anophelinae, Culicinae, and Toxorhynchitinae. There are 38 genera of mosquitoes, 34 of which are in the subfamily Culicinae. Culicines are divided into 10 tribes; the most diverse tribes in terms of numbers of genera and species worldwide are Aedini and Sabethini. Fourteen genera from North America are formally described, and the number of species in each, are *Anopheles* (16), *Aedes* (7), *Ochlerotatus* (69), *Psorophora* (15), *Haemagogus* (1), *Culex* (29), *Deinocerites* (3), *Culiseta* (8), *Coquillettidia* (1), *Mansonia* (2), *Orthopodomyia* (3), *Wyeomyia* (4), *Uranotaenia* (4), and T*oxorhynchites* (1) (Woodbridge and Walker 2002).

Although 3500 mosquito species have been described worldwide, relatively few are significant vectors of human diseases. Goddard (2007) listed 22 medically important mosquito species from the United States (Table 1.1).

Table 1.1 Medically important Mosquitoes in the U.S. (Goddard 2007)

Mosquito Species	**Disease Agent**
Aedes aegypti	Dengue (DG), Yellow fever (YF)
Ae. albopictus	DG, YF, California group encephalitis (CE)
Ochlerotatus dorsalis	Western equine encaphalitis (WEE)
Oc. melanimin	WEE, CE
Oc. nigromaculis	WEE, CE
Oc. sollicitans	Eastern equine encaphalitis (EEE)
Oc. taeniorhynchus	Venezuelan equine encaphalitis (VEE), CE, West Nile virus (WNV)
Oc. triseriatus	CE, WNV
Oc. trivittatus	CE
Ae. vexans	CE, EEE, WNV
Anopheles crucians complex	VEE, EEE
An. freeborni	Malaria (M), WEE, St. Louis encephalitis (SLE)
An. quadrimaculatus complex	M, WNV
Coquillettidia perturbans	EEE, VEE
Culex nigripalpus	SLE, WNV
Cx. pipiens/quinquefasciatus	WNV, SLE, WEE, VEE
Cx. restuans	WNV, EEE, WEE
Cx. salinarius	WNV, EEE
Cx. tarsalis	WEE, WNV, SLE
Culiseta inornata	WEE, CE
Mansonia titillans	VEE
Psorophora columbiae	VEE, EEE, WNV

Important Mosquito Transmitted Disease Agents

Mosquitoes are a major public health problem worldwide and are estimated to transmit agents of diseases to more than 70 million people annually (Fradin 1998). The major human diseases agents for which transmitted by mosquitoes are malaria, eastern equine encephalitis, Japanese encephalitis, La Crosse encephalitis, St. Louis encephalitis, West Nile virus, western

equine encephalitis, dengue fever, Rift Valley fever, yellow fever, Murray Valley encephalitis, O'Nyong-nyong, Ross River, Sindbis, and filariasis (Eldridge et al. 2000).

Among more than 520 arboviruses registered in the International Catalogue of Arthropod-Borne Viruses, less than half have biological relationships with mosquitoes, and about 100 infect humans (Woodbridge and Walker 2002). The most significant arboviruses causing human diseases belong to four genera within three families: 1) *Togaviridae* (Alphavirus), 2) the *Flaviviridae* (Flavivirus), 3) *Bunyaviridae* (Bunyavirus and Phlebovirus); some of these arboviruses infect both humans and domestic animals and cause illness in both (Woodbridge and Walker 2002).

The *Flaviviridae* contains eight antigenic complexes and many unassigned viruses including 70 types, subtypes, and varieties throughout the world (Woodbridge and Walker 2002). The most important mosquito-borne flaviviruses are: yellow fever virus, dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, louping ill virus, Powassan virus, Wesselsbron virus, and Rocio virus (Eldridge et al. 2002). Yellow fever virus was the first arbovirus ever isolated and it is the prototype of the genus Flavivirus. Walter Reed demonstrated that mosquitoes transmitted yellow fever virus in 1901. Subsequently, Murray Valley encephalitis virus (Formerly named as Australian X disease) was isolated in 1917. The next Flavivirus to be discovered, St. Louis encephalitis virus, was isolated during an outbreak in Illinois in 1933. In 1937, WNV was isolated from a febrile woman from the West Nile Province of Uganda in Africa (Sfakianos 2005). WNV was not associated with encephalitis until 1951 in Egypt when WNV was isolated from the brain of a horse with encephalitis (Sfakianos 2005).

West Nile Virus

West Nile virus belongs to the St. Louis encephalitis complex (Eldridge et al. 2000). Giladi et al. (2001) reported that the 1999 WNV epidemic in the U.S. originated from the introduction of a stain that had been circulating in Israel. The way this strain was introduced into the U.S. is not known. When genomic sequences of WNV isolates from the New York outbreak were compared with various WNV strains, the highest similarity (≥99.8) was found with a WNV strain from a goose that died in the 1998 Israeli epizootic (Lanciotii et al. 1999). Beasley et al. (2002) compared genetic and neurovirulence properties of 19 strains of WN virus, including 2 from North America, and observed significant differences in their neuroinvasive phenotype in mice and hamsters that correlated with virus genotype. The virus (NY99-4132 strain) isolated in North America was found to be highly neuroinvasive (Brault et al. 2004).

WNV infection outcomes vary due to several factors, including virus strain, dose and route of inoculation, and the age, genetic susceptibility, and immune status of the host (Sfakianos 2005). According to public health officials, even with extremely aggressive strains of WNV, most infected people do not show any symptoms (Abramovitz 2004). The 20 percent of infected patients who show symptoms after the incubation period are separated into two groups. The first group displays mild symptoms, including fever, headache, body aches, and sometimes nausea, vomiting, a rash, and swollen lymph nodes. This relatively mild syndrome is called West Nile fever. Symptoms do not last long and generally disappear with no lasting effects (Sfakianos 2005). Individuals in the second group of patients with more severe symptoms are generally diagnosed with West Nile encephalitis, West Nile meningitis, or West Nile meningoencephalitis. The patients present with varying symptoms depending on which part of the brain is inflamed;

symptoms may include headache, fever, neck stiffness, stupor, disorientation, coma, tremors, convulsions, muscle weakness and paralysis (Abramovitz 2004).

The range of symptoms of WNV infection from asymptomatic to fatal encephalitis is similar in horses and humans. However, the proportion of infected individuals that develop encephalitis is much higher in horses (Komar 2003). Studies indicate that 10% of the infected horses show clinical symptoms including ataxia, weakness of limbs, recumbency, muscle fasciculations, fever, paralysis or drooping lip, tritching face or muzzle, teeth grinding and blindness. Most veterinarians recommend that all horses be vaccinated against WNV.

Avian hosts also show a spectrum of clinical symptoms when they are infected with WNV. Individuals from one-hundred eighty bird species had been reported with fatalities due to WNV infection through 2002 (Komar 2003). Certain avian species, such as corvids, have been found to be very susceptible to WNV. WNV infected birds generally show signs of lethargy, recumbency, and in some cases are hemorrhagic.

Recent studies (Miller et al. 2003; Steinman et. al. 2003; Klenk and Komar 2003) have demonstrated that WNV infects amphibians and reptiles. West Nile virus was first reported to infect American alligators (*Alligator mississippiensis*) in the United States in 2001, when WNV-associated deaths occurred in 250 alligators in commercial alligator houses in Georgia (Miller et al. 2003). In 2002, more than 1,000 WNV-associated alligator deaths were recorded in Georgia, and there were similar reports from alligator farms in Florida (Miller et al. 2003; Jacobson et al. 2005). In 2003, more than 700 WNV-associated hatchling deaths occurred at three Louisiana alligator farms (ProMed-mail, 2003).

West Nile Virus Historical Data

In 1937, scientists first recognized WNV in the blood sample of a febrile woman in Uganda's West Nile District (Eldridge and Edman 2000). The virus became known as West Nile Fever in North Africa and the Middle East during the 1950s, and was occasionally isolated from febrile children (Hayes et al. 2005). The first indication that the virus could cause central nervous system infection was when elderly people were diagnosed with WNV encephalitis in Israel in 1957. In the early 1960s, WNV equine encephalitis was first observed in Egypt and France. The first serological evidence of WNV in Turkey was documented in 1970's where the virus was found in humans and sheep. Ozkul et al. (2006) have shown that ass-mules, cattle, dogs, horses and humans have also tested positive for WNV- neutralizing antibodies from 10 in different provinces from Turkey. In 1974, South Africa had the largest known human outbreak of WNV, with approximately 10,000 cases. From 1996 to 2000, fatal cases of human and equine encephalitis occurred in Romania, Morocco, Tunisia, Italy, Russia, Israel and France (Zeller and Schuffenecker 2004). West Nile virus was also detected in human, horses and birds during the 1990s from Algeria, Romania, Czech Republic, Volgograd, Russia and the Congo (Hubalek et al. 1999; Komar 2003). West Nile encephalitis was also reported in horses in Italy in 1998 and in France in 2000 (Hubalek et al. 1999).

The first case of WNV recorded in the Western Hemisphere was from a dead American crow in New York City in 1999 (Lanciotti et al. 1999). Although scientists remain unclear about how it was introduced, the New York 1999 (NY99) strain of the virus was identical to one isolated from a dead goose in Israel in 1997 (Zeller and Schuffenecker 2004). Sixty-two human cases of WNV including seven deaths were recorded in New York City in 1999, and the virus rapidly spread to other states (Lanciotti et al. 1999; Kulasekera et al. 2005).

The CDC reported information through the WNV Surveillance System that indicated an increase in the geographic range of WNV activity in 2000 compared with 1999. Due to rapid expansion of the virus, 17 states along the Atlantic and Gulf coasts, New York City, and the District of Columbia conducted WNV surveillance, and began to monitor mosquitoes, sentinel chicken flocks, wild birds, and potentially susceptible mammals. In 1999, WNV was detected only in Connecticut, Maryland, New Jersey, and New York (CDC, 2000). In 2000, epizootic activity in birds and/or mosquitoes was reported from Connecticut, Delaware, Maryland, Massachusetts, New Hampshire, New Jersey, New York, North Carolina, Pennsylvania, Rhode Island, Vermont, and Virginia and the District of Columbia.

The largest human outbreak of WNV in the United States included 4,156 reported cases in 2002, 329 of which were in Louisiana (Zohrabian et al. 2004). In 2002, 44 states and the District of Columbia reported WNV activity, and 39 states and the District of Columbia had reported human cases (Huhn et al. 2003). In 2002, WNV cases also were reported in the Cayman Islands, Mexico, El Salvador, Jamaica, the Dominican Republic, and Guadeloupe in humans, horses and resident birds (Franco et al. 2003; Quirin et al. 2004; Zeller and Schuffenecker 2004). West Nile virus activity was first found in birds and mosquito pools in Canada in 2001. The first confirmed human cases of WNV were reported in 2002 (PHAC, 2006).

Louisiana's subtropical location with favorable habitats for mosquitoes, lying in the Mississippi flyway favors the enzootic WNV life cycle (Gleiser et al. 2007). West Nile virus surveillance first began in Louisiana in the spring of 2000 because rapid expansion of the virus was expected. WNV spread to Louisiana faster than predicted and the first WNV positive Blue Jay was reported in July 2001 (Baldwin and Navarre 2005). Nine equine cases from southern parishes and one human case from Jefferson Parish were reported soon there after.

In 2002, 329 human WNV cases were reported from Louisiana from at least 23 parishes, and 50 in East Baton Rouge (EBR) Parish (Godsey et al. 2005; Gleiser et al. 2007). In 2002, a total of 1,247 birds were tested (43 species) from 56 sites in EBR Parish and 7 species were found WNV positive. The East Baton Rouge Mosquito and Rodent Control (EBRMARC) tested 242 blood serum samples from 38 sentinel chickens during the 2002 WNV outbreak and the first WNV positive chicken was observed on June 24th, coinciding with the onset of the first human case. An increased incidence of WNV exposure of chickens in St. Tammany Parish mirrored an increase in human cases at about the same time (Palmisano et al. 2005). West Nile virus was detected from mosquito pools two weeks prior to the spike of human cases in EBR Parish. Of the 50 human cases reported from EBR Parish, 41 had one or more mosquito pools sampled per case site, and 37 had one or more birds sampled per case site, and one or more samples from each of these sites tested positive for WNV.

In 2002, Louisiana reported the highest number of WNV human cases to date and Zohriban et al. (2004) indicated that costs attributable to WNV epidemic were substantial. Zohriban et al. (2004) showed that the costs from June 2002 to February 2003 attributable to the 2002 WNV epidemic in Louisiana were $20.1 million. This was likely an underestimate, since some of the costs associated with illness or public health response were not available.

Louisiana public health authorities reported fewer WNV cases and associated deaths in 2003 than in 2002, 124 human cases and of 7 deaths. The WNV human case count was 4 in EBR Parish in 2003. The general pattern indicated that there was an increase of cases in north Louisiana compared to 2002. In 2003, the first WNV suspected cases in alligators were reported in October. Of the four alligator farms reporting sick animals, two had imported hatchlings from

Florida, one from Texas, but the fourth had locally hatched alligators. Nevarez et al. (2005) tested all suspected cases and found them WNV RNA positive.

State health officials reported 114 human cases and 7 deaths in 2004 (LA DHH, 2005). There were fewer cases but the same number of deaths as in 2003. Both 2003 and 2004 had noticeably lower reported infections than in 2002. In 2005, a total 171 human cases of WNV occurred in Louisiana, 25 from EBR Parish (CDC, 2006). The number of human cases was higher compared to 2003 and 2004, but Louisiana Department of Health and Hospitals reported no significant increase due to hurricanes Katrina and Rita (LA DHH, 2006). Authorities also stated that the majority of cases occurred in north Louisiana, which was not directly affected from hurricanes. In 2006, the WNV human case count was 180 and EBR Parish reported 15 cases (CDC, 2006).

West Nile Virus Virology

WNV particles are spherical and approximately 50 nm in diameter, consisting of a unit membrane and dense core (Deubel et al. 2001). The WNV genome is 11,000-12,000 nucleotides long and encodes seven non-structural proteins and three structural proteins. The nucleocapsid contains a single stranded RNA. The positive stranded RNA is packed within the core protein C (Lindenbach and Rice 2003). The surface of the virus is composed of the envelope (E) and membrane (M) proteins. Envelope protein, capsid protein and membrane protein are the structural proteins. The NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 are proteins of WNV that do not contribute to the overall structure of the virion. The E glycoprotein is the major antigenic determinant on WNV particles and has important biological roles such as virion assembly, cell receptor recognition, fusion with cell endosomal membranes, agglutination of red blood cells, and induction of B and T cell responses associated with protective immunity (Deubel et al.

2001). During the maturation of nascent virus particles within the secretory pathway, the M protein is produced (Lindenbach and Rice 2003).

West Nile Virus Replication

Flaviviruses replicate in a variety of cells of vector and host species. Flavivirus specific cell receptors have not been identified. Presumably, virus binding to the cell may be promoted through the initial interaction of E protein with heparan sulfate residues present on the surface of an insect, mammal, or avian cells (Deubel et al. 2001). The WNV genome has a single open reading frame encoding for one polyprotein that is cleaved cotranslationally and posttranslationally at specific sites by host and viral proteases. This is the way the virus produces the virion and replicase components (Deubel et al. 2001).

Once the virus has gained entry into a cell and unpacked its RNA, replication begins with the synthesis of a negative stranded RNA. Negative-stranded RNA serves as a template for the future synthesis of positive stranded genomic RNA (Lindenbach and Rice 2003). Replication occurs in the perinuclear region of the endoplasmic reticulum. The numbers of immature virions increase within the membrane-bound vesicles and are transported through the secretory pathway to the cell membrane. When furin cleaves precursor M (prM) protein, mature virus leaves the cell by exocytosis (Deubel et al. 2001).

West Nile Virus Epidemiology

West Nile virus transmission cycles generally occurs between mosquitoes and birds in nature. However, studies showed that WNV host range appears quite broad including mammals and reptiles (Komar et al. 2001; Ludwig et al. 2002; Nevarez et al. 2005; Bentler et al. 2007). Wild and domestic birds stand out from other vertebrates because they develop viremia of

sufficient duration and magnitude to infect vector mosquitoes (Komar 2003). For example, mean peak viremia titers in American Crows exceed $9x10^9$ PFU/mL in sera (Komar et al. 2003). The first report of WNV infection in domestic birds was reported in Israel in 1997-1999, in young geese (McLean 2002). Hooded Crows and House Sparrows showed high antibody prevalence against WNV in Egypt and researchers were able to isolate the virus from naturally infected Hooded Crows (Telford et al. 1955). West Nile virus has been isolated from some migrating species including, Barred Warbler in Cyprus and the Turtle Dove in Slovakia (Rappole et al. 2000). The virus was first isolated in the New World in New York City in 1999 from a dead American Crow (Lanciotti et al. 1999). Birds are heavily exposed to WNV in the U.S. During the 1999 WNV outbreak, large die-offs of wild and captive birds occured at the Bronx Zoo. Komar et al. (2001) sampled a total of 430 birds' representing 18 species' and 33% were found to have neutralizing antibody against WNV. Certain species such as geese, chickens, House Sparrows, and Rock Doves were frequently infected with WNV in the New York area. House Sparrows appeared to be an important reservoir host for WNV in northeastern Queens. Kilpatrick et al. (2006) suggested that American Robins may be the most important amplification host for WNV in urban and residential areas in the eastern USA. Common passerine birds including Northern Cardinal, House Sparrow, Blue Jays and Mockingbird were reported as principal amplifying hosts for WNV in Slidell, St. Tammany Parish, Louisiana (Komar 2005); the Carolina Wren and Red-bellied Woodpecker also were infected with WNV in high frequencies.

An incidental host, also known as a dead-end host, does not develop viremia capable of transmission of WNV to other organisms. Mammals, such as humans and horses, are incidental hosts of WNV. It has been repeatedly reported that the viremias developed in most mammals are not high enough to efficiently infect mosquitoes; observed levels of WNV viremia in

experimentally infected cats, dogs and horses seldom reach or exceed 10^4 PFU/mL (Bunning et al. 2002; Austgen et al. 2004).

Bronx Zoo/Wildlife Convervation Park reported the WNV activity among the mammal collection in 1999 (Ludwig et al. 2002). A total of 35 species representing 18 families among the mammals were tested for WNV. Indian elephant, Indian rhinoceros, ring-tailed lemur, red panda, snow leopard, and babirusa (pig-deer) serum samples were positive for WNV infection. West Nile virus positive dogs and horses were reported from the boroughs within New York City in 1999 (Komar 2001). High prevalence rates of antibodies to WNV were detected in mesopredators including opossums, raccoons, striped skunks from California, Arizona, Texas, Louisiana, Ohio, and Wyoming during 2003 and 2004 serosurvey studies (Bentler et al. 2007). Following the 2002 WNV outbreak in Louisiana, researchers collected blood samples from captive rhesus monkeys, pigtail macaques and baboons that were permanently housed outdoors at a facility located at St. Tammany Parish, Louisiana. These three species showed antibodies against WNV infection, however no compatible clinical illness or neurologic disease was observed in any of the animals (Ratterree et al. 2003).

Vector Incrimination and Vector Competence

Arthropod-borne virus (arbovirus) diseases have 3 components in their natural transmission cycle. The first component is the virus which is a biological agent that can cause the disruption of the normal physiology of its vertebrate host. The host is generally a vertebrate animal, and Eldridge (2000) defined infection as the establishment of a virus in a host. The third component is the vector, which is the organism that transmits the pathogen from host to host.

It is important to establish the role that particular arthropod species plays in the transmission of a particular infectious disease agent. No arthropod species can be incriminated as a vector until several requirements are satisfied (Barnett 1962).

1) Demonstration that members of suspected arthropod species feeds upon a vertebrate host, or otherwise makes effective contact with the host under natural conditions.
2) Demonstration of a convincing biological association in time and/or space between the suspected vector species and clinical or subclinical infections in vertebrate hosts.
3) Repeated demonstration that the suspected vector species, collected under natural conditions, harbors the identifiable, infective stage of the infectious agent.
4) Demonstration of efficient transmission of the identified infectious agent by the suspected vector species under controlled experimental conditions (establishing vector competence).

It is important to satisfy the above requirements for vector incrimination because efficient disease prevention and control activities depend upon vector control. Vector incrimination must be evaluated at the population level of the suspected vector species. For example, Goddard et al. (2002) reported various WNV infection and transmission values from different populations of *Culex. p. pipiens* L. and of *Cx. p. quinquefasciatus* Say mosquitoes collected from California. Even sympatric populations of a mosquito species may vary in vector competence for arboviruses as well as allopatric populations (Hardy et al. 1976). Vaidyanathan and Scott (2007) reported two different populations of *Cx. p. quinquefasciatus* from Los Angeles and Riverside

Counties that were equally susceptible to WNV infection. The authors indicated that seven days after infective feeding neither sample transmitted WNV; however, 14 days after infective feedings, samples from Riverside County were infected and transmitted WNV, but samples from Los Angeles County were not infected and did not transmit the virus.

Almost any bloodfeeding arthropod which feeds upon a viremic vertebrate host can obtain and retain the virus for some time, but that does not necessarily mean that the arthropod is a competent vector (Barnett 1962). Vector competence refers to the ability of individuals in a population of arthropods to acquire, maintain, and transmit a given strain of pathogen. Establishing vector competence is one of the requirements for vector incrimination.

West Nile Virus Transmission Cycle

Studies from 1952 to 1954 in Egypt provided the first documented observations of the transmission cycle of WNV. Wild birds contract WNV, and following the initial infection, the virus spreads when mosquitoes that have taken a bloodmeal from an infected bird feed on other animals (Abramovitz 2004). Birds are the primary amplification hosts for WNV and the primary vectors are mosquitoes. However, WNV also has been isolated from ticks; ticks have been shown to transmit the virus in laboratory experiments (Eldridge and Edman 2000). Recently, Sabio et al. (2006) reported WNV RNA positive *Culicoides stellifer* (Coquillett) in Louisiana.

WNV has been isolated from over 40 mosquito species (most in the genus *Culex*) in Africa, southern Europe and western Asia. The most significant species for different geographical areas are as follows: *Cx. tritaeniorhynchus* Giles in south Asia, *Cx. annulirostris* Skuse in Australia, *Cx. perexiguus* Theobald (formerly *Cx. univittatus*), in North Africa and the Middle East, *Cx. univittatus* complex in sub-Saharan Africa, and Old World forms of *Cx. pipiens* and *Cx. quinquefasciatus* (Komar 2003).

Since 1999, individuals from 62 mosquito species have been found to be WNV RNA positive in the United States. *Ae. vexans*, *Cx. restuans*, *Cx. salinarius*, and *Cx. pipiens* were found to be infected with WNV during 1999. The following year virus was isolated from 10 more mosquito species. In the United States, WNV infection has been reported by CDC to be associated with 62 different species including: *Aedes aegypti* L., *Ae. albopictus* Skuse, *Ae. atlanticus/tormentor* Dyar and Knab, *Ae. atropalpus* Coquillett, *Ae. Canadensis* Theobald, *Ae. cantator* Coquillett, *Ae. cinereus* Meigen, *Ae. condolescens* Dyar and Knab, *Ae. dorsalis* Meigen, *Ae. dupreei* Coquillett, *Ae. fitchii* Felt and Young, *Ae. fulvus pallens* Wiedemann, *Ae. grossbecki* Dyar and Knab, *Ae. infirmatus* Dyar and Knab, *Ae. japonicus* Theobald, *Ae. melanimon* Dyar, *Ae. nigromaculis* Ludlow, *Ae. provocans* Walker, *Ae. sollicitans* Walker, *Ae. squamiger* Coquillett, *Ae. sticticus* Meigen, *Ae. stimulans* Walker, *Ae. taeniorhynchus* Wiedemann, *Ae. triseriatus* Say, *Ae. trivittatus* Coquillett, and *Ae. vexans* Meigen*; Anopheles atropos* Dyar and Knab, *An. barberi* Coquillett, *An. crucians/bradleyi* Wiedemann, *Anopheles franciscanus* McCracken, *An. freeborni* Aitken, *An. hermsi* Barr and Guptavanij, *An. punctipennis* Say, *An. quadrimaculatus* Say, and *An. walkeri* theobald; *Coquillettidia perturbans* (Walker) ; *Cx. apicalis* Adams, *Cx. coronator* Dyar and Knab, *Cx. erraticus* Dyar and Knab, *Cx. erythrothorax* Dyar, *Cx. nigripalpus* Theobald, *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans* Dyar, *Cx. salinarius* Coquillett, *Cx. stigmatosoma* Dyar, *Cx. tarsalis* Coquillett, *Cx. territans* Walker, and *Cx. thriambus* Dyar; *Culiseta incidens* Thomson, *Cu. Impatiens* Walker, *Cu. Inornata* Williston, *Cu. Melanura* Coquillett, and *Cu. morsitans* Theobald; *Deinocerites cancer* Theobald; *Mansonia tittilans* Walker; *Orthopodomyia signifera* Coquillett; *Psorophora ciliata* Fabricius, *Ps. columbiae* Dyar and Knab, *Ps. ferox* Humboldt, *Ps. howardii* Coquillett ; *Uranotaenia*

sapphirina Osten Sacken (CDC, 2007). Sixty-four mosquito species have been characterized from Louisiana (Fox 2006), and of those, 35 have been shown to be infected with WNV.

Turell et al. (2001) showed that *Cx. salinarius* is a highly efficient vector of WNV. Molaei et al. (2006) indicated that its physiologic competence to transmit WNV, high infection rates in nature and seasonal distribution that overlaps with human cases, all indicate that *Cx. salinarius* is a bridge vector of WNV in the northeastern United States. Goddard et al. 2002 reported that *Cx. erythrothorax*, *Cx. pipiens*, *Cx. stigmatosoma*, and *Cx. tarsalis* species are highly efficient laboratory vectors. Sardelis et al. (2002) showed that *Ae. albopictus* is an efficient laboratory vector of WNV and may function as a bridge vector.

Sardelis et al. (2002) indicated that *Cx. quinquefasciatus* and *Cx. nigripalpus* are competent but only moderately efficient vectors. However, *Cx. quinquefasciatus* and *Cx. nigripalpus* are considered to be the primary enzootic and epizootic vectors of WNV in the southeastern United States (Godsey et al. 2005). Turell et al. (2000) found *Ae. vexans* to be only moderately efficient as an laboratory vector. Sardelis et al. (2001) reported that *Cq. perturbans* was an inefficient WNV vector in laboratory studies, however this species is considered as a potential WNV vector due to its bird feeding behavior.

Transmission of WNV generally occurs due to horizontal transmission. However, within mosquitoes there are other modes of virus transmission such as vertical and venereal transmission. Complete vertical transmission can occur either when the virus is passed from the female vector to the next generation or when infected male sperm fertilizes eggs laid by non-infected females (Edman 2000). Reisen et al. (2006) demonstrated that *Culex* females can be infected venereally, however those females did not amplify virus after mating and the eggs of a single female that retained WNV for 3 d were WNV negative. There are several recent studies

supporting the role of vertical transmission for WNV maintenance. Miller at al. (2000) first reported vertical transmission for WNV in nature from *Cx. univittatus* (Theobald) males collected from Rift Valley Province of Kenya. Reisen et al. (2006) demonstrated vertical transmission of WNV in *Cx. pipiens* complex by testing field collected male mosquitoes and larvae in California. Phillips and Christensen (2006) detected WNV from field collected 3rd and 4th stage *Cx. erythrothorax* Dyar larvae in Utah. Rosen (1988) first reported vertical transmission of flavivirus for Japanese and St. Louis Encephalitis viruses by *Aedes* mosquitoes. Additionally, *Ae. albopictus* also found to vertically transmit WNV (Baqar et al. 1993).

Surveillance Methods for West Nile Virus

Surveillance for mosquito-borne diseases involves an organized monitoring and sampling system. An efficient surveillance program requires an understanding of the biology, ecology and interactions of the vertebrate hosts and mosquito vectors (Moore et al. 1993). The goal of the surveillance program is to provide risk assessment for human arboviral infection (Day et al. 2003) and reliable surveillance tools that predict positive human cases are needed. For a functional surveillance program, combinations of surveillance tools may be used and there are several tools that have been developed to monitor arboviruses. Some methods target mosquito populations and some of them target hosts such as such as birds, horses, and humans.

For WNV surveillance, mosquitoes are collected from trapping locations, sorted, and identified as to species. Commonly used traps are Centers for Disease Control (CDC) light traps and gravid traps (Reiter, 1983) baited with fish oil emulsion (Alaska Fish Fertilizer® Lilly Miller Brands, Clackamas, OR). The mosquitoes must be stored at –80° C until they are tested for arbovirus. Mosquitoes are placed in pools of 1-50 mosquitoes and tested for the presence of WNV. Mosquito testing is a common surveillance method and many mosquito abatement

districts use infection rates as their primary surveillance method to predict human cases in their area (Vaeth et al. 2007).

Historically, sentinel chickens have been used for arbovirus surveillance in the U.S. Chickens are placed in high risk infection locations (generally in shaded areas) where they can be exposed to mosquitoes. Blood samples are taken weekly and tested for seroconversion by plaque-reduction neutralization test (PRNT). A seropositive chicken is considered to be proof of virus activity.

The use of dead birds in WNV surveillance is a new tool that began to be used in 1999. Prior to WNV introduction in the United States, there was no mosquito-borne virus in the U.S. that caused high mortality rates in birds (Day et al. 2003). Dead birds were the first indication of WNV introduction to a new area in North America (Day et al. 2003). Dead birds are reported by citizens, a mosquito control district, or health department employees. New York City used observations of the density of dead crows in 2001, and found that the areas with high dead crow density (DCD – measured in dead crows per square mile) in the early season were significantly more likely to have human cases of WNV infection (Eidson et al. 2005). The dead bird surveillance method is passive. The success of the method depends on the public to find and report dead birds and takes a long time to achieve results. This method does not provide proof of local transmission but it is still being used by many local authorities (Cooke et al. 2006; Ward et al. 2006).

Free-ranging wild birds, especially passerine species are important vertebrate hosts of WNV. Thus, wild birds can be repeatedly sampled to test for antibodies or virus. Adult and immature birds are captured and banded and a blood sample is taken (Day et al. 2003). Generally, virus activity and antibody seroprevalence in birds are well correlated with the risk of

human cases. However, antibodies can persist in the birds for 2 years or longer, so a positive adult bird does not always provide information about the present virus activity (Moore et al. 1993). Seropositive nestling birds provide information on more recent infections.

When a human outbreak of arbovirus activity is anticipated, surveillance in humans can be increased. In this case, all encephalitis and meningoencephalitis admissions should be screened for major arboviral diseases (Moore et al. 1993). The most important goal of human case surveillance may be to determine the geographic areas that need more surveillance and control efforts. Human cases of arbovirus related encephalitis are often the only indication of virus circulating in a given area (Moore et al. 1993). This is a passive surveillance method similar to dead bird reporting. In addition to humans, reported arbovirus cases in horses also can be used as a surveillance tool. Horse breeders, owners and veterinarians are encouraged to report sick horses in WNV prevalent areas (Abramovitz 2004).

WNV is a disease of humans and domestic animals that requires reporting to the CDC. The Centers for Disease Control and Prevention (CDC) collect surveillance data on human cases, dead birds, horses, sentinel chickens, mosquito pools from the states through ArboNET. ArboNET is a web-based surveillance network including fifty-four state and local public health departments. ArboNET data are used to track the epidemic temporally and geographically and to direct public health resources to activities such as reducing mosquito populations (Huhn et al. 2003).

Virus Detection Protocols for West Nile Virus in Mosquito Specimens and Vertebrate Hosts

Enzyme- linked immunosorbent assay (ELISA) is a direct binding assay for antibody or antigen. It is the first and most commonly used, commercial test for WNV detection (Sfakianos

2005). The ELISA uses antigen-antibody specificity to detect the WNV infection in serum, and if antibody specific for a defined antigen is present, the antibody binds to the antigen. Nonspecific absorption is blocked and unbound antigens are washed away (Janeway et al. 2001). Antibody-antigen complex is detected using an enzyme and when the enzyme solution is added to the well, it binds to the complex. Bound enzyme then acts on a color indicator and color change indicates that the animal has been probably exposed to WNV (Sfakianos 2005).

Vero cell plaque assays are standard virus detecting techniques. In this assay, monolayers of cultured cells are incubated and then covered with nutrient medium containing a supplement (commonly agar) that results in the formation of a gel (Flint et al. 2000). Double agar overlay is used to detect live WNV in the mosquito pools. Supernatant of the homogenized mosquito pools is inoculated into confluent Vero cell mono layers (Nasci et al. 2002). After a one hour incubation at 37° C, cells are overlayed with warm agar and then maintained at 37° C. If WNV exists in the samples, the Vero cells form plaques or clear areas where the cells have lysed. Plates are observed for plaques for 10 days. When a single infectious virus is sufficient to form a plaque, the titer of the virus is calculated by counting plaque-forming units (Flint et al. 2000). The identity of the virus detected in Vero cell assay has to be confirmed by using different techniques such as Reverse transcriptase-polymerase chain reaction (RT- PCR).

Polymerase chain reaction (PCR) assay was first used for WNV RNA detection during the 1990's (Porter et al. 1993), and has later replaced by RT- PCR. RT- PCR is based on the 5'-3' exonuclease activity of the Taq DNA polymerase (Leutenegger 2001). The forward primer has a probe with two fluorescent dyes. One is a reporter dye, 6-carboxyfluorescein (FAM) and its emission spectra quenched to the second fluorescent dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). As the polymerase extends the primer, the probe is displaced and the nuclease

activity in the polymerase cleaves the reporter dye from the probe. When the reporter dye is released, a fluorescent signal is generated. Then an optical detection system sends the data to computer software (Sequence Detection System). The data collected from PCR amplification is still in exponential phase. The exponential phase is identified as cycle threshold (CT). Pools are considered positive when they are less than 37 (Naugle et al. 2004). The CT value is directly associated with the amount of PCR product. The ABI Prism 770 is a laser-coupled spectrophotometer and it monitors the position of the 96-well microtitre plate every 8 minutes (Leutenegger, 2001). At the end of the forty cycles all the data for analysis are stored in the Sequence Detection System (SDS) file. RT- PCR has a major advantage over the other techniques due to increased sensitivity of fluorescent dye-labeled probes.

Nucleic Acid Sequence Based Amplification (NASBA) is a robust amplification technology that has been used to detect a number of pathogens, including WNV. The amplification process uses three enzymes, including reverse transcriptase, T7 RNA polymerase, and RNase H. The amplified RNA product can be detected in real time through the use of molecular beacon probes included in the amplification reaction (Lanciotti and Kerst 2001). Molecular beacon has a probe with 5′ fluorescent dye and a 4-dimethylaminophenylazobenzoyl (DABCYL). These fluorescent dyes are designed to form stem-loop structures and due to the proximity of the 5′ and 3′ ends of the probe, the 5′ fluorescent dye emission spectra is quenched by the DABCYL. If the tested sample is positive for WNV RNA, the probe will hybridize to the target, separating the reporter dye from the quencher, resulting in a measurable increase in fluorescence. NASBA provides results in less than one hour, and this is definitely an advantage over RT-PCR for rapid virus detection (Lanciotti and Kerst 2001).

The Vectest® is an antigen panel assay designed by Medical Analysis Systems, Inc. to detect WNV and SLE antigens in mosquito pools (Nasci et al. 2002). The assay uses type-specific monoclonal antibodies against WNV and SLE antigens. According to the manufacturer's guidelines, mosquito pools containing up to 50 individual mosquitoes are ground up using a grinding solution (Ryan et al. 2003). Following this step, the test strips are placed into the test tubes. If WNV and/or SLE are present, the antigen will bind to the specific antibodies on the test strip producing a reddish purple line. The assay only shows if the antigen is present, it does not provide any quantitative assessment of viral antigen in the sample. Vectest is a less sensitive virus screening method compared to RT- PCR (Nasci et al. 2002).

The Rapid Analyte Measurement Platform (Ramp®) WNV test, is designed to provide rapid, easy and reliable diagnostic information. The Ramp test uses similar principles to detect the virus antigen. A Ramp test uses antibodies bound to fluorescently labeled latex particles (The Vectest uses gold sol particle labels) (Stone et al. 2005). The Ramp test strips are inserted into a reader which calculates the ratio between fluorescence emitted at the test and control wavelength zones. Results are displayed as RAMP units. Stone et al. (2005) reported that RAMP is more sensitive than Vectest for testing dead corvid oral swab samples.

All the assays that are used for WNV RNA detection from mosquito specimens and vertebrate hosts can be efficient assays, but all of them exhibit some kind of limitations. Due to the establishment of WNV throughout the United States, surveillance programs expanded and required an increased number of mosquitoes to be processed. As a result, automated, rapid RT-PCR procedures for detecting WNV RNA replaced standard cell culture assays for live virus (Lanciotti et al. 2000). In addition, the TaqMan RT-PCR is the most sensitive assay compared to other assays and now the most preferred tools for screening large numbers of mosquito pools in

surveillance programs (Lanciotti et al. 2000). Vero cell assay is also very sensitive, and has the added benefit of being able to detect any virus that will grow in the cell culture, but this assay also is slow and expensive. Commercially available dipstick test for detecting WNV antigen in mosquito pools are also available, and VecTest has the advantage of getting rapid results even though is not as sensitive as RT- PCR assay. Panella et al. (2005) also indicated that Vectest® has the potential to simplify dead bird surveillance for WNV by reducing required resources such as specialized equipment and costly reagent kits needed to achieve rapid and accurate results. The Vectest® assay is less sensitive than RT-PCR and Vero cell assay, but is relatively inexpensive, doesn't require costly equipment or specially trained personnel (CDC, 2003).

CHAPTER 2: EVALUATION OF SURVEILLANCE METHODS FOR DETECTION OF WEST NILE VIRUS ACTIVITY IN EAST BATON ROUGE PARISH, LOUISIANA, 2004-2006

Introduction

West Nile virus (WNV) is a mosquito-borne pathogen that was first isolated in Uganda in 1937 from the blood of a woman with fever (Zohrabian et al. 2004). The first human case of WNV in the United States occurred in New York City (NYC) in 1999 (Duebel and Zeller 2001). Public health authorities in New York City started a WNV surveillance program using sentinel chicken flocks in 2000 (Lukacik et al. 2006). However, the first seroconversions of sentinel chickens were observed in August while the first human case occurred on 20 July 2000 (Komar 2001). Subsequently, New York public health authorities began to use the density of dead crows (DCD, dead crows per square mile), because counties with high dead crow densities in the early season were significantly more likely to have a human case of WNV disease (Eidson et al. 2005).

From 1999 to 2002, WNV spread to most regions of the continental United States. Following the rapid spread of the virus, different public health-oriented agencies had to select among different surveillance protocols and tools for WNV activity (White 2001). Public health authorities have a wide variety of surveillance methods available to use to monitor arbovirus activity in the U.S. (White 2001). These surveillance methods include mosquito testing, horse surveillance, sentinel chickens, wild bird serum survey, dead bird reports, and human case reports. In general, mosquito abatement districts use mosquito testing as their primary surveillance tool for WNV activity (Buena et al. 2007; Veath et al. 2007).

Sentinel birds have been used to monitor western equine encephalitis (WEE), eastern equine encephalitis (EEE), and St. Louis encephalitis (SLE) in many states including, Alabama, California, Florida, Louisiana, Maryland, Massachusetts, New Jersey, New York, Tennessee,

Texas, Ohio, Michigan, Indiana, and Illinois for decades (Komar 2001). In contrast to NYC, the sentinel chickens surveillance program in Florida provides important data about WNV activity prior to horse and human cases (Blackmore et al. 2003).

Following the introduction of the virus into Louisiana in 2002, public health authorities began to monitor virus activity using sentinel chickens and other methods. The purpose of this study was to determine if testing mosquitoes or chickens from modified sentinel chicken boxes for WNV would provide information for early warning of WNV activity prior to human cases. In one year, we also compared the effectiveness of Centers for Disease Control (CDC) light traps baited with CO_2, gravid traps, and sentinel chicken box traps for collecting WNV positive mosquitoes.

Materials and Methods

Experimental Design

In 2004, 2005, and 2006, four sentinel chicken box traps (SCBT) were placed in four different sites in East Baton Rouge (EBR) Parish. The sentinel chicken box traps were 1.22 x 2.44 x 1.22 m wooden boxes holding two to four caged chickens; one of the ends of each box was left open. In 2004, sites were selected by EBR Parish Mosquito Abatement and Rodent Control (EBRPMARC) personnel in areas with previous WNV human cases. There were two urban locations (Site I and Site II) and two suburban locations (Site III and Site IV). Site I (30.49223N-91.16052W) was an open location with a very little vegetation. Site II (30.45578N-91.00609W) was an open area with mixed vegetation. Site III (30.45578N- 91.12119W) was a small farm, and Site IV (30.57262N-91.07115W) was a wooded area intersected by several large ditches. In 2005 and 2006, Site I (30.38097N-91.20696W) was a horse activity center with wooded areas and open pasture, and Site II (30.56209N-9111533W) was an urban residence with

mixed vegetation, while sites III and IV remained the same. In 2004, SCBT remained in the study sites the entire year, while the SCBT were in the sites from May to November during 2005 and 2006.

Each site was visited once per week throughout the study for mosquito sampling. Mosquitoes were collected using a backpack aspirator (Bioquip Products, Inc. Rancho Dominguez, CA, USA) and then immediately were transferred to emergence traps (Bioquip Products, Inc. Rancho Dominguez, CA, USA) which were labeled by date and site. Using emergence traps allowed separation of live mosquitoes from those that were dead. Dead mosquitoes were discarded and the emergence traps were held in a freezer at –20° C to kill the live mosquitoes. Mosquitoes were separated on a chill table (Industrial Inventions, Inc. West Windsor, NJ, USA) according to their species and placed into groups of males, bloodfed females and non-bloodfed females. Non-bloodfed females and males were placed into cryovials in pools of 1-50 mosquitoes and stored at –80° C until tested for the presence of WNV. The head and the thorax of the bloodfed mosquitoes were removed and stored at –80°C until being tested for WNV RNA. Dissecting probes were treated with a bleach solution to prevent cross contamination among samples.

Sentinel chickens were bled weekly from May to the middle of October in all three years of the study by EBRPMARC personnel. The blood samples were placed into 2 ml Microtainer Serum Separator Tubes (Becton Dickinson # VT365956 Franklin Lakes, NJ, USA) and stored in a small ice chest in the field. Then blood samples were stored in a refrigerator at 25 °C until shipment to the Louisiana Animal Disease and Diagnostic Laboratory (LADDL). The samples were centrifuged and tested by either an immunoglobulin M (IgM) or IgG ELISA. When the results indicated a positive chicken, that sentinel chicken was retested. If the retest also was

positive, that sentinel chicken was removed from the field and replaced with a naïve chicken. The human case data for 2004 to 2006 was obtained from the Office of Public Health of Louisiana (http://www.dhh.louisiana.gov/offices/page.asp).

In 2005, in addition to the sentinel chicken box traps, mosquitoes also were collected with CDC light traps baited with CO_2, and gravid traps (Reiter, 1983) baited with fish oil emulsion (Alaska Fish Fertilizer® Lilly Miller Brands, Clackamas, OR). The traps were placed 100 meters apart at each of the four sites and each trap was rotated weekly among the three trapping locations at each study site. All non-bloodfed females were sorted and stored as described above. In 2006, gravid traps also were maintained in the study sites by EBRPMARC personnel, who sorted and then stored non-bloodfed female mosquitoes at –80° C before testing for the presence of WNV.

Mosquito Pool Testing

All mosquito pools were tested at the LADDL by RT-PCR for presence of WNV RNA. Mosquitoes were homogenized in 1 ml of BA-1 diluent with copper-coated steel beads (Lanciotti et al. 2000). Mosquitoes were homogenized using a TissueLyser (Qiagen, Valencia, CA, USA), for 4 min at 25 Hz, and then homogenates were centrifuged for 2 min at 5,796 x g. A volume of 220 µl of cleared homogenate was used for RNA extraction. RNA extraction was performed using Qiagen QIAamp® Virus Biorobot® 9604 Kit (Qiagen, Valencia, CA, USA). Briefly, homogenates were mixed with 240 µl AL buffer and 40 µl of protease, and then incubated at 60° C for 10 min. After the addition of 265 µl of 100% ethanol, samples were transferred to QIAamp® 96 plate (Qiagen, Valencia, CA, USA) and subject to three washes, RNA was eluted from the Qiagen columns in a volume of 86 µl elution buffer. Centrifugation at 5,796 x g was

used to perform washes and elute. Elute was stored at -20° C until testing (Lanciotti et al. 2000; Eisler et al. 2004).

Real-time RT-PCR was performed by using QuantiTect Probe RT-PCR Kit (Qiagen, Valencia, CA, USA) with 15 pmol of each primer, 3 pmol of probe, and 5 µl of eluted RNA in a 15 µl total reaction volume. Primer sequence forward 5´TCAGCGATCTCTCCACCAAAG3´ and primer sequence reverse 5´GGGTCAGCACGTTTGTCATTG3´ were used to amplify the envelope gene (Lanciotti et al. 2000). The WNV RNA was detected as an increase in the fluorescence of the probe FAM-5´TGCCCGACCATGGGAGAAGCTC3´-BHQ1. The samples were subjected to 45 cycles of amplification in an ABI 7900HT real time PCR instrument (PE Applied Biosystems). The following cycling times and temperatures were used: 1 cycle of 48° C for 30 min and 95° C for 10 min followed by 45 cycles of 95° C for 15 sec, and 60° C for 1 min. Samples were interpreted as positive if the cycle threshold (CT) units were less than 40.

Statistical Analysis

An Analysis of Variance (ANOVA) was used to compare the number of mosquito species caught in different trap types for four experiment sites for the 2005 sentinel chicken surveillance study (SAS Institute 2001). A least significant difference (LSD) test was used to detect significant differences between the sites and trap types. Mosquito infection rates were determined by calculating the maximum likelihood estimate (MLE) using a computer based program with 95% confidence intervals (http://www.cdc.gov/ncidod/dvbid/westnile/ software.htm).

Results

In 2004, a total of 2,689 specimens (334 mosquito pools) representing 10 mosquito species, were collected in sentinel chicken box traps and tested for WNV (Table 2.1). West Nile

virus was detected in 21 pools of females of three mosquito species: *Culex quinquefasciatus* Say, *Anopheles quadrimaculatus* Say and *Psorophora ferox* (von Humboldt) (Table 2.1). Two pools of male *Culex. spp*. also were positive for WNV. The first WNV positive mosquitoes were collected on 13 May 2004, and the first human cases in EBR Parish were reported during the 27th CDC week (27 June-3 July). The first sentinel chicken seroconversion for WNV was detected on 19 July. Thirteen chickens developed detectable antibody to WNV from 19 July to 11 October (Fig. 2.1).

Table 2.1 Total number of mosquitoes collected from four different sites in EBR Parish with sentinel chicken box traps, 2004-2006.

	2004			2005			2006		
Species	**No mosq**	**No Pools Tested**	**No Pools WNV+**	**No mosq**	**No Pools Tested**	**No WNV+ Pools**	**No mosq**	**No Pools Tested**	**No WNV+ Pools**
Cx. quinquefasciatus	1915	188	14	1537	131	5	3310	99	3
Cx. restuans	115	0	0	0	0	0	0	0	0
Cx. erraticus	50	29	0	0	0	0	48	12	0
Cx. nigripalpus	15	5	0	0	0	0	0	0	0
Cx. salinarius	31	15	0	0	0	0	0	0	0
Ae. albopictus	3	0	0	3	1	0	0	0	0
An. punctupennis	8	4	0	0	0	0	0	0	0
An. quadrimaculatus	134	24	4	45	8	0	109	28	0
An. crucians	24	11	0	6	0	0	0	0	0
Ps. ferox	12	5	3	0	0	0	0	0	0
Cq. perturbans	0	0	0	0	0	0	1	1	0
Cx. spp (males)	382	53	2	849	43	1	654	39	1
Total	2689	334	23	2440	183	6	4121	179	4

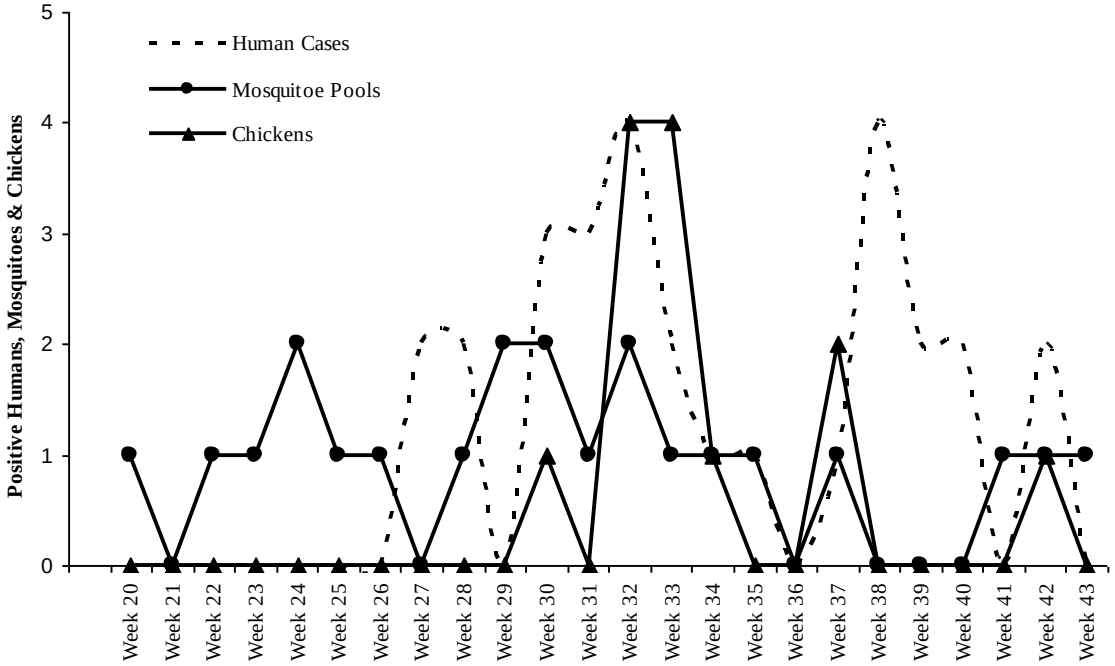

Fig 2.1 WNV activity in EBR Parish from CDC week 20 through CDC week 43 in human cases, mosquito pools and sentinel chickens (2004).

In 2005, a total of 2,440 specimens of 3 mosquito species in 183 pools were tested for WNV (Table 2.1). West Nile virus was detected in five pools of female *Cx. quinquefasciatus* and one pool of *Culex. spp*. males (Table 2.1). The first WNV positive mosquitoes were collected on 21 May 2005, and the first human cases in EBR Parish were reported during the 27th CDC week (26 June-2 July). The first sentinel chicken seroconversion for WNV was detected on 12 July; 22 chickens developed detectable antibody to WNV from 12 July to 4 October (Fig. 2.2).

In 2006, a total of 4,121 specimens (179 mosquito pools) representing four species were collected (Table 2.1). West Nile virus was detected in three pools of female *Cx. quinquefasciatus* and one pool of *Culex. spp*. males (Table 2.1). West Nile virus positive mosquitoes were collected on 4 July 2006 both from sentinel chicken box traps and gravid traps, and the first human cases in EBR Parish were reported during 29th CDC week (16 July-22 July); 17 chickens developed detectable antibody to WNV at four sites from 11 July to 5 September (Fig. 2.3).

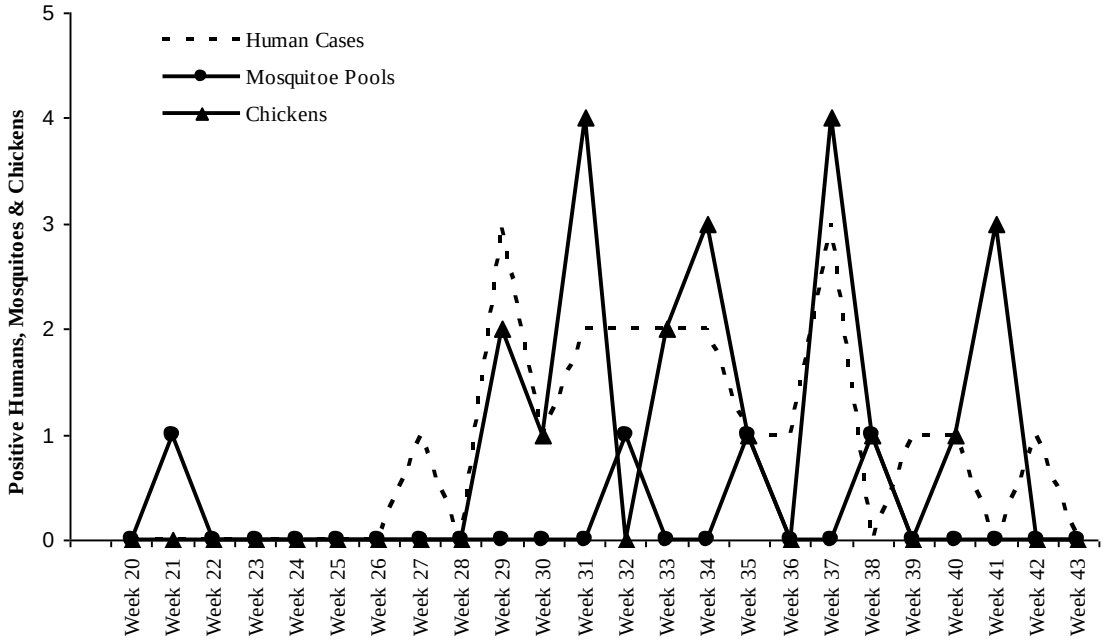

Fig 2.2 WNV activity in EBR Parish from CDC week 20 through CDC week 43 in human cases, mosquito pools and sentinel chickens (2005).

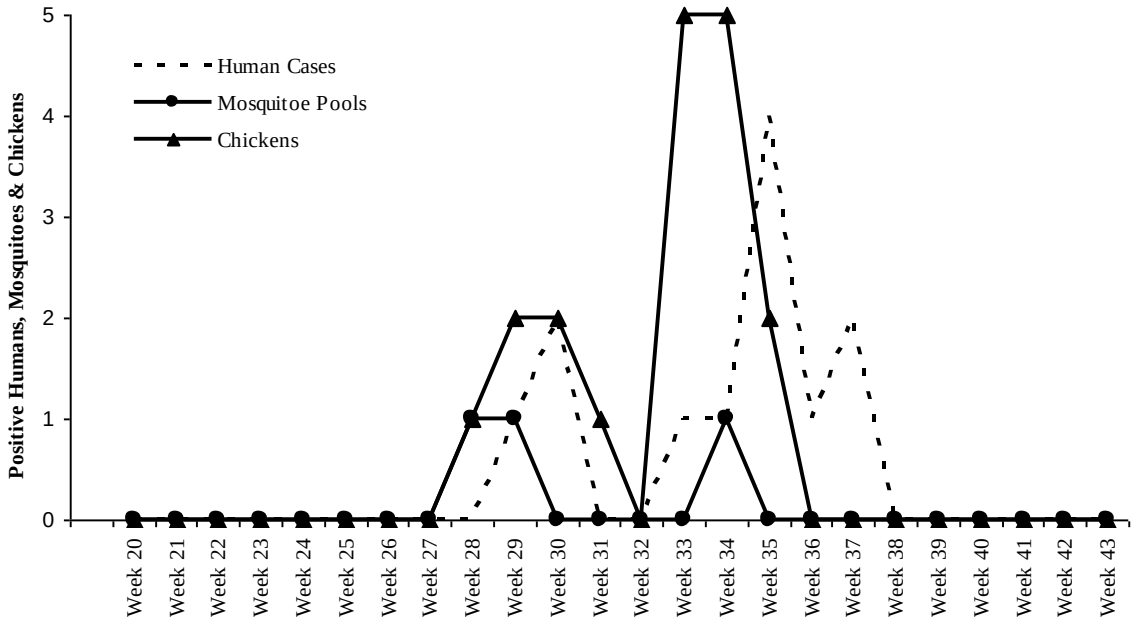

Fig 2.3 WNV activity in EBR Parish from CDC week 20 through CDC week 43 in human cases, mosquito pools and sentinel chickens (2006).

In 2005, the most abundant species collected in all three traps was *Cx. quinquefasciatus*: CDC light trap 32.63%, gravid trap 94.8 %, and sentinel chicken box trap 63.14% (Table 2.3). In addition to the species listed in Table 2.3, small numbers of *Culex coronator* Dyar and Knab, *Cx. salinarius* Coquillett, *Cx. erraticus* (Dyar and Knab), *Coquillettidia perturbans* (Walker), *Culiseta inornata* (Williston), *Ochlerotatus triseriatus* (Williston), *Mansonia tittilans* (Walker), *Ps. colombiae* (Dyar and Knab), and *Ps. ciliate* (Fabricius) individuals also were collected from the CDC light traps. There was no statistically significant difference between gravid traps and sentinel chicken box traps in the number of species caught ($F = 110.6$; $df = 9$; $P = 0.888$). However, CDC light traps caught significantly more species than the other two traps ($F = 110.6$; $df = 9$; $P < 0.0001$). We detected six WNV positive pools from sentinel chicken box traps (6/184), 28 WNV positive pools from gravid traps (28 /303), and one positive pool from CDC light traps (1 /145). The proportion of WNV detections from gravid traps were significantly higher than those from CDC light trap and sentinel chicken box trap in 2005 ($\chi 2 = 18.01$, $p < 0.0001$).

At site IV, we collected WNV positive male mosquitoes on 5 May, 2004, one week before the first WNV positive *Cx. quinquefasciatus* females were collected on 13 May, 2004. Similarly, in 2005 we collected WNV positive male mosquitoes on 11 July and the first WNV positive *Cx. quinquefasciatus* females were collected on 17 July. In 2006, we collected WNV positive male mosquitoes and WNV positive *Cx. quinquefasciatus* females from Site IV on the same date (2 August 2006).

A total of 1,222 pools containing 19,353 mosquito specimens collected between 2004-2006, representing 18 species, were analyzed for the presence of WNV RNA. For 2004, 2005

Table 2.2 WNV detections from mosquitoes collected in East Baton Rouge Parish in four different sites, 2004-2006.

Year	Species	No. Infected Pools	No. Pools Tested	Trap Type	Infection Rate (95% Confidence Interval) [a]
2004	*Cx. quinquefasciatus* (Site III)	7	106	SCB Trap[b]	6.13 (2.7 – 11.9)
	An. quadrimaculatus (Site III)	2	25	SCB Trap	24.78 (4.4 – 80.5)
	Cx. quinquefasciatus (Site IV)	5	81	SCB Trap	12.56 (4.8 – 27.1)
	Ps. ferox (Site III)	1	1	SCB Trap	c
	Ps. ferox (Site II)	2	5	SCB Trap	4.30 (0.78-13.90)
	Cx. spp. (males) (Site III)	1	25	SCB Trap	5.90 (0.31-29.46)
	Cx. spp. (males) (Site IV)	1	28	SCB Trap	5.24 (0.31-25.20)
2005	*Cx. quinquefasciatus* (Site I)	13	60	Gravid Trap	7.85 (4.4 – 13.3)
	Cx. quinquefasciatus (Site II)	1	20	CDC Trap	2.08 (0.12 – 10.1)
	Ae. vexans (Site II)	1	20	CDC Trap	2.08 (0.12 – 10.1)
	Cx. quinquefasciatus (Site II)	1	37	SCB Trap	2.94 (0.18 – 14)
	Cx. quinquefasciatus (Site III)	4	51	Gravid Trap	1.82 (0.59– 4.37)
	Cx. quinquefasciatus (Site III)	3	39	SCB Trap	8.45 (2.3 – 22.2)
	Cx. quinquefasciatus (Site IV)	11	90	Gravid Trap	3.30 (1.7 – 5.7)
	Cx. quinquefasciatus (Site IV)	1	41	SCB Trap	1.43 (0.08 – 7.06)
	Cx. spp. (males) (Site IV)	1	23	SCB Trap	1.61 (0.09-7.90)
2006	*Cx. quinquefasciatus* (Site III)	1	26	SCB Trap	0.83 (0.05 – 4)
	Cx. quinquefasciatus (Site II)	2	19	SCB Trap	5.18 (1 – 16.4)
	Cx. spp. (males) (Site IV)	1	13	SCB Trap	2.74 (0.17-13.13)

[a]Estimated number of infected females per 1,000; determined by MLE.
[b]Sentinel Chicken Box (SCB).
c MLE values can not be calculated because one mosquito pool tested for *Ps. ferox* from Site IV, and that pool was the only WNV positive pool.

and 2006, the average pool sizes of *Cx. quinquefasciatus* collected from sentinel chicken box traps were 5.7, 18.5 and 18.7, respectively. The majority of WNV positive pools were from *Cx. quinquefasciatus* and MLE ranged from 0.83/1,000 to 12.56/1,000 (Table 2.2). The highest infection rate was found in *An. quadrimaculatus* (24.78/1,000).

Table 2.3 Total number of mosquitoes collected in East Baton Rouge Parish in four different sites using CDC light traps, Sentinel chicken box traps and gravid traps, 2005

Species	CDC Light Trap	Sentinel Chicken Box	Gravid Trap
Cx. quinquefasciatus	438	1537	9513
Cx. nigripalpus	26	0	0
An. punctupennis	6	0	2
An. quadrimaculatus	20	45	0
An. crucians	0	6	0
Ae. vexans	628	0	0
Ae. albopictus	192	3	206
Ps. ferox	27	0	4
Cx. spp (males)	5	849	315
Total	1342	2440	10040

Discussion

In this study, sentinel chickens were not useful as an early warning of human risk for WNV. The first seroconversions in sentinel chicken flocks were detected after the onset of human cases. These results are consistent with findings in NYC (Cherry et al. 2001; Komar 2001). Palmisano et al. (2005) also reported that WNV infected sentinel chickens peaked at about the same time as human cases in St. Tammany Parish, LA. Unlike the sentinel chicken surveillance results from New York and Louisiana, Blackmore et al. (2003) reported that sentinel chicken seroconversions were the first indication of WNV activity in Putnam and Leon counties in Florida, where they detected WNV positive human cases 6.5 wk after the first WNV positive chicken. In California, sentinel chicken seroconversion for WNV was detected 7.5 weeks prior to human case onset in Los Angeles county, and in Santa Barbara 4 weeks before the first human case in 2005. Similar results were reported in 2006 from Imperial and Los Angeles counties suggesting that sentinel chicken surveillance is a useful predictor of WNV activity prior to human cases in California (CDHS, 2007).

The difference in the value of sentinel chickens as an early warning system in different states may be related to the different WNV vectors in these locations. In New York, *Culex pipiens* L. is considered to be the primary vector of WNV (Lukacik et al. 2006), and *Cx. quinquefasciatus* is considered to be the primary vector in Louisiana (Godsey et al. 2005). *Culex tarsalis* Coquillett is considered to be the primary vector of WNV in California (Goddard et al. 2002), and *Culex nigripalpus* Theobald is considered to be the primary vector in Florida (Godsey et al. 2005). Different mosquito species in different locations may vary in vector competence for WNV transmission (Goddard et al. 2002; Vaidyanathan and Scott 2007). For example, *Cx. tarsalis* has been determined to be one of the most efficient vectors of WNV tested from North America (Turell et al. 2000; Sardelis et al. 2001). Goddard et al. (2002) found that *Cx. tarsalis* was the most efficient laboratory vector among other *Culex* species they tested from California. Goddard et al. (2002) also exposed different *Culex* species to low doses of WNV in the laboratory; *Cx. tarsalis* was the only species in which positive transmission was detected following 7 days of incubation.

Another possible reason why sentinel chicken seroconverisons did not provide an early warning for the presence of WNV activity in this study might be related to the number of sentinel chicken flocks in the field and the number of mosquitoes being drawn to the chickens (Tabachnick 2006). Both Florida and California, which have successful sentinel chicken surveillance programs as an early warning for WNV activity, have high numbers of sentinel chickens flocks in the field compared to New York and Louisiana. New York public health authorities monitored 99 sentinel chickens in 2000 and Louisiana public health authorities monitored 38 chickens in 2002 (Cherry et al. 2000; Gleiser et al. 2007). Florida public health authorities monitored 2,128 sentinel chickens in 2001, and California public health authorities

monitored 2,120 sentinel chickens in 2003 for WNV antibodies (Blackmore et al. 2003; Reisen et al. 2004). In addition to the high numbers of sentinel chickens tested, California and Florida have the advantage of having had extensive arbovirus surveillance programs for a long time, which allows for the selection and continuous use of sites that historically have shown arbovirus activity.

In the first two years of the present study, mosquito collections from sentinel chicken box traps tested WNV positive in May. In 2006, the first WNV positive mosquitoes were collected from sentinel chicken box traps in July. In 2004, 2005 and 2006, mosquitoes were collected from sentinel chicken box traps tested WNV positive 7, 6, and 2 wk prior to human cases, respectively (Fig. 2.1, Fig. 2.2, Fig 2.3). Our results indicate that testing mosquitoes collected in sentinel chicken box traps might be a better early predictor of human cases than seroconversions of sentinel chickens, which would be expected due to the period between exposure to an infected mosquito and seroconversion for chickens.

In 2005, gravid traps collected more WNV infected mosquitoes (in most cases *Cx. quinquefasciatus*) than CDC light traps or sentinel chicken box traps at each sampling site. However, WNV positive mosquitoes were collected in sentinel chicken box traps earlier than gravid traps and CDC light traps. In 2006, WNV positive mosquitoes were collected from sentinel chicken box traps and gravid traps on the same date. Lukacik et al. (2006), conducted a mosquito surveillance study in New York State (2004-2006), using both CDC light traps and gravid traps. Although 71.9% of all mosquito pools were from CDC light traps, 67.7% of all WNV positive pools came from gravid traps. Lukacik et al. (2006) concluded that gravid traps were superior to CDC light traps as surveillance tools for the collection of WNV infected mosquitoes, particularly for *Cx. pipiens*. Reisen et al. (2004) also reported that most of the WNV

positive mosquito pools contained specimens of *Cx. quinquefasciatus* collected from gravid traps compared to CDC light traps. Therefore, gravid traps may be more efficient than CDC light traps for virus surveillance in the southeastern United States; these traps collect a higher proportion of parous *Cx. quinquefasciatus* than light traps (Reisen et al. 2004). Since *Cx. quinquefasciatus* appears to be the primary enzootic and epidemic vector in the southeastern United States, knowledge of the infection rates in *Cx. quinquefasciatus* is important to assess the risk of human infection (Sardelis et al. 2001). Although we found the best trap method for collecting infected *Cx. quinquefasciatus* females was the gravid trap, which is consistent with the studies by Lukacik et al. (2006) and Reisen et al. (2004), we detected WNV in *Cx. quinquefasciatus* females collected from the sentinel chicken box traps earlier than in *Cx. quinquefasciatus* females collected from the gravid traps.

The reason for collecting WNV positive mosquitoes from sentinel chicken box traps before gravid traps could be related to collecting vertically infected host-seeking nulliparous female mosquitoes in the SCBT (Nasci et al. 2001; Dhom et al. 2002; Medlock et al. 2005). In all 3 years of the study, we collected WNV positive male mosquitoes before or on the same date as collecting WNV positive females, suggesting that the first WNV positive females collected were vertically infected. The possibility that the first WNV positive females were vertically infected also is supported by the similar MLE's of *Cx. quinquefasciatus* females and *Cx. spp* males from Site III and Site IV.

The goal of this study was to identify a surveillance tool to detect WNV activity prior to human infection. In all three years, we detected WNV activity in mosquito collections from sentinel chicken box traps prior to the onset of human cases, while there were no seroconversions in the chickens prior to human cases. Sentinel chicken box traps and gravid traps primarily

collected *Cx. quinquefasciatus* mosquitoes which are considered to be the primary enzootic and epidemic vectors of WNV in the Louisiana. In this study, approximately 6-fold more *Cx. quinquefasciatus* were caught in the gravid traps than sentinel chicken box traps. Processing high numbers of mosquitoes for WNV detection can be labor intensive and expensive. Since we detected WNV activity by testing the mosquitoes from the sentinel chicken boxes, testing mosquitoes collected from sentinel chicken box traps may be an economical way to monitor for early WNV activity.

CHAPTER 3: IDENTIFICATION OF BLOODMEAL ORIGINS FOR MOSQUITOES COLLECTED AT ALLIGATOR FARMS IN LOUISIANA

Introduction

West Nile virus was first reported to infect American alligators (*Alligator mississippiensis*) in the United States in 2001, when WNV-associated deaths occurred in 250 alligators in commercial alligator houses in Georgia (Miller et al. 2003). In 2002, more than 1,000 WNV-associated alligator deaths were recorded in Georgia, and there were similar reports from alligator farms in Florida (Miller et al. 2003; Jacobson et al. 2005). In 2003, more than 700 WNV-associated hatchling deaths occurred at three Louisiana alligator farms (ProMed-mail, 2003).

Alligator farming is an important agricultural component of the economy of certain states. According to an economic impact report on the Marsh Market Program in 2005, there were 61 alligator farms in Louisiana which produced more than half a million alligators. The Marsh to Market concept was initiated in 1972 as a conservation tool in Louisiana to protect alligator populations, preserve critical wetland habitats, and provide economic benefits (NGN, 2001). In 2005, the alligator farm harvest value in Louisiana was $33 million. The potential of WNV infection in captive American alligators could severely affect producers in Louisiana. Therefore, it is critically important to know how WNV is introduced into and spreads among captive populations of alligators.

In the U.S., all confirmed cases of WNV infections of alligators have been reported in farmed alligators housed under controlled conditions. Once an initial WNV infection is established in the housed alligators, contaminative transmission between alligators may occur. Transmission among alligators through fecal shedding of virus has been suggested to be the

primary mechanism in the spread of WNV infection in farmed alligators (Briese and Bernard 2005).

West Nile virus can be introduced into captive alligators through the introduction of infected alligators or infectious food. In Georgia, the initial cases of WNV infections in alligators were associated with feeding of WNV- infected horsemeat (Miller et al. 2003). In Idaho, 600 farmed alligators died in 2003 due to WNV infections (ProMED-mail, 2003). The farm had obtained one-month old hatchling alligators from a farm in Florida, and since WNV had been not detected in Idaho in 2003, state officials concluded that the alligators were infected in Florida prior to shipment (ProMED-mail, 2003).

The first suspected WNV cases in alligators in Louisiana were reported in August, 2003. Of the four alligator farms reporting sick animals, three had imported hatchlings from Florida or Texas, but the fourth farm had locally hatched alligators (Nevarez et al. 2005). The alligator farms were not using any food sources that could have been contaminated with WNV, which pointed to the possibility that the route of infection might have been mosquito-borne. Specific recommendations for reducing the risk of WNV transmission in commercial alligator production systems will require a detailed understanding of the role of mosquitoes as vectors of WNV. If mosquitoes are capable of introducing WNV into captive alligator populations in Louisiana, then practicing mosquito control measures could be an important way to reduce the risk of WNV epizootics.

Many studies have examined the vector competence of different mosquito species to transmit arboviruses to reptiles (Hayes et al. 1964; Whitney et al. 1968, Cupp et al. 2004), and to transmit WNV to birds and mammals (Austgen et al. 2004; Komar et al. 2003). However, there have been no studies on the competence of mosquitoes to transmit WNV to alligators. In order to

incriminate mosquitoes as vectors of WNV for farmed alligators, an association between mosquitoes and alligators will be required. An accepted method for showing an association between vectors and hosts is vector bloodmeal identification. The purpose of this study was to identify the origin of the bloodmeals of mosquitoes collected at commercial alligator farms in Louisiana.

Materials and Methods

Experimental Design

Field studies were conducted at three alligator farms in Louisiana, two of which had previously had WNV infections in alligators. Alligator farm A was located in East Baton Rouge Parish, alligator farm B was located in Terribonne Parish, and alligator farm C was located in Tangipahoa Parish. Alligator farm A (30.370167 N, 90.975631 W) was located close to a suburban residence area with mixed vegetation intersected by several large ditches. Alligator farm B (29.5795N- 90.82505W) was surrounded by swampland. Alligator farm C (30.46193N, 90.53501W) was located close to a suburban residence area with mixed vegetation.

Centers for Disease Control (CDC) light traps with either incandescent (alligator farm A) or ultraviolet light (alligator farm A, B and C), gravid traps (Reiter, 1983) baited with fish oil emulsion (Alaska Fish Fertilizer® Lilly Miller Brands, Clackamas, OR), backpack aspirators (Bioquip Products, Inc., Rancho Dominguez, CA), and resting boxes were used to collect mosquitoes. The CDC light traps were located inside the alligator houses at alligator farm A and B, but were located only outside the alligator houses at alligator farm C. Gravid traps and resting boxes were placed in areas with vegetation. The resting boxes were 30.5x30.5x30.5 cm wooden boxes, painted black on the outside and red on the inside. Backpack aspirators were used to collect engorged female mosquitoes from resting boxes and from vegetation. Traps were placed

at farms A and B once per week in the afternoon and collections were made the following morning. Collections were made at alligator farm A from 28 July 2004 to 22 November 2005 and from 4 April 2006 to 9 June 2006. Collections made at alligator farm B were from 23 August 2005 to 25 October in 2005 and from 4 April 2006 to 14 November 2006. Collections were made at alligator farm C two to three times per month from 3 September 2004 to 3 November 2004 and from 12 June 2005 to 28 September 2005.

Mosquito Processing

Mosquitoes were placed in containers marked with trap number, date, and location and transported to the laboratory in an ice chest containing dry ice. In the laboratory, mosquitoes were separated on a chill table (Industrial Inventions, Inc. West Windsor, NJ, USA) according to their species and placed into groups of bloodfed females, non-bloodfed females, and males. The abdomens of the bloodfed mosquitoes were removed, placed individually into cryovials, and stored at –80° C for bloodmeal identification. The rest of the bodies of the bloodfed females were stored individually at –80° C and later tested for the presence of WNV. Dissecting probes were sterilized with a bleach solution to prevent cross contamination among samples.

DNA Extraction for Sequence

DNA was extracted from bloodfed mosquitoes using the tissue protocol of the QIAmp DNA mini kit (Qiagen, Valencia, CA). Briefly, the sample was mixed with 180 µl ATL buffer and 20 µl protease and incubated at 56°C overnight. The following day, 200 µl 100% ethanol was added. After this step, samples were transferred to spin columns. After the two washes in the spin column, DNA was eluted with 50 µl AE buffer. Samples were stored in –70 °C until the PCR assay was conducted.

PCR Primers and Conditions

The unlabeled primers (cytb) BM1 (5'-CCC CTG AGA ATG ATA TTT GTC CTC A), and BM2 (5'- CCA TCC AAC ATC TCA GCA TGA TGA AA (Integrated DNA Technologies, Coralville, IA) were used to amplify a portion of the cytochrome B gene. HotStarTaq Master Mix Kit (QIAGEN, Valencia, CA) was used for PCR reactions. Each 30µl reaction included 1xPCR buffer, 200 µM each dNTP, 1.5 mM MgCl2, 0.2 mM of each primer, 1.25 U of TaqDNA polymerase, and 1.0 µl of template. Amplification was performed using an iCycler (Bio-Rad, Hercules, CA). The thermal cycling conditions consisted of 15 min 95°C, 36 cycles at 95°C for 30 sec, 60°C for 50 seconds, 72°C for 40 seconds, and a final extension for 5 minutes at 72°C. The PCR products were stained with ethidium bromide and visualized on an agarose gel.

Sequencing of Cytb-Derived PCR Products of Known Vertebrates

All PCR products that produced a band on the gel were used for cycle sequencing after purification. Polyethylene glycol precipitation method was used to clean the samples (Ausubel et al. 2002). The cycle sequence reactions were prepared using Big-Dye Terminator mix (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol using 0.16 µM unlabeled BM1 primer. The PCR products were purified using the DyeEx 96 removal kit (Qiagen) before sequencing. The sequence data were compared with GenBank database using the basic local alignment search tool (BLAST) program (NCBI).

Results

The bloodmeals of 403 field-collected, bloodfed mosquitoes (representing 14 species) were processed. There was no PCR product for 14 samples, and a match from known profiles was not found for 157 samples following the sequencing. A match of ≥97 percent was made for

237 (58.8%) bloodmeals; 156 (65.8%) of the bloodmeal sources were mammalian, 56 (23.6%) were avian, 24 (10.1%) were reptilian, and 1 (0.4%) was amphibian.

A total of 178 bloodfed female mosquitoes representing 9 species were collected from alligator farm A. Of the 178 bloodfed mosquitoes, 38 were from CDC light traps, 34 were from gravid traps, 78 were from resting sites, and 10 were from resting boxes. The majority (71%) of the bloodfed specimens collected were *Culex quinquefasciatus* Say. Of the 126 bloodmeals of *Cx. quinquefasciatus* that were identified, 10.3% were derived from alligators, 23.8% were from birds, and 65.8% were from mammals. Alligator blood was detected in mosquitoes of three species; 13 *Cx. quinquefasciatus* were identified as having fed on alligators (Table 3.1). We detected avian bloodmeals in mosquitoes of seven species (Table 3.2). Avian blood was identified in 30 *Cx. quinquefasciatus*; 50% of the avian bloodmeals identified from *Cx. quinquefasciatus* were derived from chickens. We detected mammalian bloodmeals in mosquitoes of six species (Table 3.3). The majority (46.6%) of the mammalian bloodmeals identified were from *Cx. quinquefasciatus* and mostly from domestic cows (31%), humans (14.4%), and White-tailed deer (10.8%).

A total of 75 bloodfed female mosquitoes representing 7 species were collected from alligator farm B. Of the 75 bloodfed mosquitoes, 65 were from CDC light traps, 7 were from gravid traps, 2 were from resting sites, and 1 was from a resting box. The majority (38%) of the bloodfed specimens collected were *Culex nigripalpus* Theobald. Of the 28 bloodmeals of *Cx. nigripalpus* that were identified, 10.7% were derived from alligators, 25% were from birds, and 64.3% were from mammals. Alligator blood was detected in mosquitoes of four species; three *Cx. nigripalpus* were identified as having fed on alligators (Table 3.4). Avian blood was identified in five species of mosquitoes (Table 3.5). Avian blood was identified in seven *Cx.*

nigripalpus; 71.4% of the avian bloodmeals identified from *Cx. nigripalpus* were derived from chickens. We detected mammalian blood in mosquitoes of five species (Table 3.6). Mammalian blood was detected in 18 *Cx. nigripalpus*; the majority of the mammalian meals identified from *Cx. nigripalpus* were derived from White-tailed deer (22.2%), domestic cows (19%), and humans (11.1%).

Table 3.1 Mosquitoes identified as having fed on an alligator (*Alligator mississippiensis*) at alligator farm A, EBR Parish, Louisiana, 2004-2006

Alligator Farm A		
Mosquito species	Collection date	Trap type
Aedes vexans (Meigen)	11/24/04	CDC light trap(outside)
Coquillettidia perturbans (Walker)	08/12/04	Aspiration
Cx. quinquefasciatus	08/25/04	Aspiration
Cx. quinquefasciatus	08/25/04	Gravid trap
Cx. quinquefasciatus	09/01/04	CDC light trap (inside)
Cx. quinquefasciatus	09/08/04	Gravid trap
Cx. quinquefasciatus	09/08/04	Aspiration
Cx. quinquefasciatus	10/13/04	CDC light trap (inside)
Cx. quinquefasciatus	11/23/04	Resting box
Cx. quinquefasciatus	12/01/04	Aspiration
Cx. quinquefasciatus	03/23/05	Aspiration
Cx. quinquefasciatus	05/25/05	Aspiration
Cx. quinquefasciatus	06/08/05	Gravid trap
Cx. quinquefasciatus	06/28/05	Aspiration
Cx. quinquefasciatus	08/04/05	Aspiration
Cx. spp	05/11/05	Gravid trap

Table 3.2 Mosquitoes identified as having fed on avian hosts at alligator farm A, EBR Parish, Louisiana, 2004-2006

Alligator Farm A		
Mosquito Species	Avian host Latin/Common name	No of BM*
Anopheles quadrimaculatus Say	*Gallus gallus*	1
Ae. vexans	*Gallus gallus*	1
Cx. quinquefasciatus	*Cardinalis cardinalis* Northern cardinal	5
Cx. quinquefasciatus	*Gallus gallus* Chicken	15
Cx. quinquefasciatus	*Zenaida macroura* Mourning dove	2
Cx. quinquefasciatus	*Piranga rubra* Summer tanager	1
Cx. quinquefasciatus	*Falco sparverius* American kestrel	1
Cx. quinquefasciatus	*Bombycilla cedrorum* Cedar waxwing	2
Cx. quinquefasciatus	*Lanius ludovicianus* Loggerhead shrike	2
Cx. quinquefasciatus	*Meleagris gallopavo* Wild turkey	1
Cx. quinquefasciatus	*Cyanocitta cristata* Blue jay	1
Cx. coronator Dyar and Knab	*Cistothorus platensis* Sedge wren	1
Culex restuans Theobald	*Zenaida macroura*	1
Cx. restuans	*Falco sparverius*	1
Culex salinarius Coquillett	*Gallus gallus*	1
Psorophora columbiae (Dyar and Knab)	*Gallus gallus*	1

* bloodmeals

A total of five bloodfed female *Cx. quinquefasciatus* were collected from alligator farm C. None of these mosquitoes had fed on alligators. One amphibian (green frog), two avian (Mourning Dove and Northern Cardinal), and two (human and domestic cow) mammalian bloodmeals were identified.

Table 3.3 Mosquitoes identified as having fed on mammalian hosts at alligator farm A, EBR Parish, Louisiana, 2004-2006

Alligator Farm A		
Mosquito Species	**Mammalian host Latin/Common name**	**No of BM***
An. quadrimaculatus	*Canis familiaris*	1
An. quadrimaculatus	*Homo sapiens*	1
Ae. vexans	*Canis familiaris*	2
Ae. vexans	*Bos taurus*	4
Ae. vexans	*Odocoileus virginianus*	1
Cq. perturbans	*Bos taurus*	1
Cx. quinquefasciatus	*Felis familiaris* Domestic cat	5
Cx. quinquefasciatus	*Canis familiaris* Domestic dog	14
Cx. nigripalpus	*Homo sapiens*	1
Cx. nigripalpus	*Didelphis virginiana*	1
Cx. quinquefasciatus	*Bos taurus* Domestic cow	26
Cx. quinquefasciatus	*Equus caballus* Horse	1
Cx. quinquefasciatus	*Didelphis virginiana* Virginia opossum	8
Cx. quinquefasciatus	*Homo sapiens* Human	12
Cx. quinquefasciatus	*Odocoileus virginianus* White-tailed deer	9
Cx. quinquefasciatus	*Myocastor coypus* Nutria	6
Cx. quinquefasciatus	*Procyon lotor* Northern raccoon	1
Cx. quinquefasciatus	*Capra hircus* Goat	1
Cx. restuans	*Canis familiaris*	1
Cx. restuans	*Bos taurus*	3
Cx. restuans	*Odocoileus virginianus*	2
Cx. salinarius	*Bos taurus*	2
Cx. salinarius	*Odocoileus virginianus*	1
Cx. spp	*Bos taurus*	1

*bloodmeals

Table 3.4 Mosquitoes identified as having fed on an alligator (*Alligator mississippiensis*) at alligator farm B, Terribonne, Louisiana, 2006

Alligator Farm B		
Mosquito species	**Collection date**	**Trap type**
Cx. erraticus (Dyar and Knab)*	10/10/06	CDC light trap (outside)
Cx. nigripalpus	09/12/06	CDC light trap (outside)
Cx. nigripalpus	09/12/06	CDC light trap (outside)
Cx. nigripalpus	10/18/06	CDC light trap (outside)
Cx. quinquefasciatus	07/28/06	Gravid trap
Cx. salinarius	04/04/06	CDC light trap (outside)
Cx. spp	10/10/06	CDC light trap (outside)
Cx. spp.	11/07/06	CDC light trap (outside)

* *Cx. erraticus* pool tested positive for WNV

Table 3.5 Mosquitoes identified as having fed on avian hosts at alligator farm B, Terribonne, Louisiana, 2006

Alligator Farm B		
Mosquito Species	Avian host Latin/Common name	No of BM*
Cx. erraticus	*Gallus gallus*	1
Cx. nigripalpus	*Gallus gallus*	5
Cx. nigripalpus	*Piranga rubra* Summer tanager	1
Cx. nigripalpus	*Cardinalis cardinalis* Northern cardinal	1
Cx. quinquefasciatus	*Gallus gallus* Chicken	1
Cx. quinquefasciatus	*Bombycilla cedrorum* Cedar waxwing	1
Cx. salinarius	*Gallus gallus*	2
Cx. spp.	*Gallus gallus*	3
Cx. spp.	*Zenaida macroura* Mourning dove	1
Ochleratatus infirmatus Dyar and Knab	*Gallus gallus*	1

*bloodmeals

Table 3.6 Mosquitoes identified as having fed on mammalian hosts at alligator farm B, Terribonne, Louisiana, 2006

Alligator Farm B		
Mosquito Species	Mammalian host Latin/Common name	No of BM*
Ae. albopictus	*Odocoileus virginianus*	1
Cx. erraticus	*Odocoileus virginianus* White-tailed deer	2
Cx. erraticus	*Didelphis virginiana* Virginia opossum	1
Cx. erraticus	*Homo sapiens* Human	2
Cx. erraticus	*Myocastor coypus* Nutria	1
Cx. nigripalpus	*Sylvilagus aquaticus* Swamp rabbit	2
Cx. nigripalpus	*Homo sapiens*	2
Cx. nigripalpus	*Philander opossum* Opossum	1
Cx. nigripalpus	*Myocastor coypus*	1
Cx. nigripalpus	*Didelphis virginiana*	1
Cx. nigripalpus	*Canis familiaris*	2
Cx. nigripalpus	*Odocoileus virginianus*	4
Cx. quinquefasciatus	*Bos taurus* Domestic cow	1
Cx. quinquefasciatus	*Myocastor coypus*	2
Cx. quinquefasciatus	*Homo sapiens*	1
Cx. nigripalpus	*Bos taurus*	5
Cx. salinarius	*Bos taurus*	2
Cx. salinarius	*Myocastor coypus*	1
Cx. salinarius	*Odocoileus virginianus*	3
Cx. spp	*Homo sapiens*	2
Cx. spp	*Canis familiaris* Domestic dog	1
Cx. spp	*Odocoileus virginianus*	3
Cx. spp	*Bos taurus*	5
Mansonia titillans (Walker)	*Myocastor coypus*	1
Ma. titillans	*Procyon lotor* Northern raccoon	1
Ma. titillans	*Bos taurus*	1

*bloodmeal

The majority (86.6%) of the alligator bloodmeals identified from mosquitoes from alligator farm A were from *Cx. quinquefasciatus*. All the specimens of *Cx. quinquefasciatus* shown to feed on alligators were collected between March and November (Table 3.1). Of the

mosquitoes identified to species and shown to feed on alligators at farm B, 50% of the alligator bloodmeals were from *Cx. nigripalpus*. All of the specimens of *Cx. nigripalpus* shown to feed on alligators were collected between September and October (Table 3.1).

Discussion

The current study was conducted at three alligator farms in Louisiana, two of which had previously had WNV infections in alligators. We detected alligator blood in 24 mosquitoes of six species from the two farms that had previous WNV infections. Nevarez (2007) had indicated that mosquitoes can take a bloodmeal from alligators by feeding at mucous membranes, between the scales, or around the eyes. Rodrigues and Maruniak (2006) showed that mosquitoes of three species (*Mansonia dyari* Belkin, Heinemann and Page, *Ma. titillans* Walker, and *Cx. erraticus*) had fed on captive alligators in Florida. However, none of these mosquito species are considered to be primary vectors of WNV in Florida. In the current study, alligator blood was detected in *Ae. vexans*, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, and *Cx. salinarius*, and many of these species are considered to be competent vectors of WNV.

Three of the species shown to have fed on alligators in the current study are not considered to be major vectors of WNV. The vector competence of *Cx. erraticus* for WNV has not been studied. However, WNV has been detected repeatedly in *Cx. erraticus* in previous studies (Hribar et al. 2004; Bolling et al. 2005; Cupp et al. 2007), which leaves the possibility that this species is a competent vector of WNV. Sardelis et al. (2001) reported that *Cq. perturbans* was an inefficient WNV vector in laboratory study, and Turell et al. (2000) found that *Ae. vexans* was a moderately efficient vector of WNV in the laboratory.

Three of the species shown to have fed on alligators in the current study are considered to be major vectors of WNV. Sardelis et al. (2002) indicated that *Cx. quinquefasciatus* and *Cx.*

nigripalpus are competent but only moderately efficient vectors. However, *Cx. quinquefasciatus* and *Cx. nigripalpus* are considered to be the primary enzootic and epizootic vectors of WNV in the southeastern United States (Godsey et al. 2005). Turell et al. (2001) showed that *Cx. salinarius* is a highly efficient vector of WNV. Molaei et al. (2006) indicated that its physiologic competence to transmit WNV, high infection rates in nature and seasonal distribution that overlaps with human cases, all indicate that *Cx. salinarius* is a bridge vector of WNV in the northeastern United States.

We were able to establish the temporal association of mosquito species feeding on alligators and reported outbreaks of WNV at alligator farms. Nevarez at al. (2006) reported WNV outbreaks at four alligator farms in Louisiana (including alligator farms A and B), and all cases were observed between August and December of 2003 (J. Nevarez personal communication). Of the six species shown to feed on alligators in this study, mosquitoes of *Ae. vexans*, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, and *Cx. quinquefasciatus*, were shown to feed on alligators between the months of August and November.

The time between an alligator being infected with WNV by a mosquito bite and the determination of an outbreak in an alligator house (tankmates infected by contaminative transmission) would be at least 12-15 days (Klenk et al. 2004). West Nile virus outbreaks were reported from alligator farm A on 26 August 2003 and from alligator farm B on 16 October 2003 (J. Nevarez personal communication). At alligator farm A, the majority of the mosquitoes shown to feed on alligators were *Cx. quinquefasciatus*, which is considered to be the primary epizootic and epidemic vector of WNV in Louisiana. Female *Cx. quinquefasciatus* were shown to feed on alligators in many months including August and September, and the timing of the WNV outbreak at farm A coincides with reported human cases in EBR Parish, where farm A is located.

The predominant mosquito species at farm B shown to feed on alligators was *Cx. nigripalpus*, and the mosquitoes shown to feed on alligators were collected between September and October, which was the exact timing for the WNV outbreak which occurred at farm B in 2003.

All of the mosquito species shown to feed on alligators in the current study also were shown to feed on birds and mammals, which supports previous reports that all of these species are opportunistic feeders (Clements 1999). Cupp et al. (2004) identified bird, mammal, and reptile bloodmeals from *Cx. erraticus* in central Alabama. Apperson et al. (2002) found that *Cq. perturbans* exhibits general feeding habits, taking bloodmeals mainly from mammals, but also from birds. Murphey at al. (1967) identified both mammal, and bird bloodmeals from *Ae. vexans*; however, there was no indication that reptiles were utilized. Bertsch and Norment (1983) indicated that *Cx. quinquefasciatus* prefers to feed avian hosts during the spring and early summer months and prefers mammalian hosts during mid-to-late summer in Mississippi. Day (2005) described *Cx. nigripalpus* as an opportunistic blood feeder, which attacks virtually any available vertebrate host in Florida. Edman (1974) indicated that *Cx. nigripalpus* is an extremely opportunistic mosquito, which feeds mainly on cattle and rabbits, and ciconiiform, passerine, and galliform birds in Florida. Murphey at al. (1967) observed that *Cx. salinarius* has non-preferential feeding on both birds and mammals, and Hayes (1961) showed limited feeding of *Cx. salinarius* on reptiles.

All of the six species which were found to take bloodmeals from alligators in this study are known to feed on birds, and many of these bird species are important amplifying hosts of WNV. For example, the Northern Cardinal is considered to be a major WNV amplification host in Louisiana (Komar et al. 2005) and Northern Cardinal bloodmeals were found in specimens of both *Cx. nigripalpus* and *Cx. quinquefasciatus* in this study.

The presence of mosquitoes, which feed on both birds and captive alligators at farms with previous WNV infections, provides strong evidence that mosquitoes may play a role in WNV transmission for captive alligators. Although mosquitoes may not feed on wild alligators, mosquitoes may be attracted to the alligator houses, because of high number of animals exhaling CO_2 and producing a large amount of organic waste that creates a warm, humid environment even during the cooler times of the year (Nevarez 2007).

Barnett (1962) suggested four requirements for vector incrimination, and we have fulfilled two of those requirements for mosquitoes as vectors of WNV for captive alligators in this study. First, mosquitoes were shown to feed on captive alligators under natural circumstances. Second, a temporal association was made between the time when mosquitoes were shown to feed on alligators and the reports of clinical infections in alligators. Another requirement to establish incrimination of mosquitoes as vectors of WNV for captive alligators would be to demonstrate that suspected vectors collected at alligator farms are WNV positive, which is the subject of Chapter 4.

CHAPTER 4: DETECTION OF WEST NILE VIRUS RNA FROM MOSQUITOES COLLECTED AT ALLIGATOR FARMS IN LOUISIANA, 2004-2006

Introduction

Between 2001 and 2003, West Nile virus (WNV) infections and associated deaths were reported in captive American alligators (*Alligator mississippiensis*) in Georgia, Florida, Louisiana, and Idaho (Miller et. al. 2003, Jacobson et al. 2005, Nevarez et al. 2005). In these southern states, alligator farming contributes to the economy of agricultural production and the potential of WNV infection in captive American alligators could severely affect producers. Therefore, it is critically important to know how WNV is introduced into and spreads among captive populations of alligators. Once housed alligators are infected with WNV, contaminative transmission between alligators may occur (Klenk et al. 2004).

Outbreaks of WNV in captive alligators have been linked to the feeding of infected meat (Miller et al. 2003) or the introduction of infected hatchlings (ProMED-mail, 2003). However, the initial source of WNV infection was not established in other outbreaks, which indicated a possibility that mosquitoes were involved in WNV transmission of housed alligators. If mosquitoes are capable of introducing WNV into captive alligator populations in Louisiana, then specific recommendations could be made to alligator producers regarding mosquito control techniques to reduce potential economical losses due to WNV related mortality and morbidity.

Until recently, there was no information to indicate that mosquitoes feed on captive alligators. Jacobson et al. (2005) reported the observation of mosquitoes feeding upon alligators at the alligator farms in Florida, but the identification of the species of the mosquitoes was not made. Subsequently, Rodrigues and Maruniak (2006), showed that 3 species of mosquitoes, *Mansonia dyari* Belkin, Heinemann and Page, *Ma. titillans* Walker, and *Cx. erraticus*, had fed on captive alligators in Florida. Recently, we have shown that mosquitoes of six species (*Ae.*

vexans, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, and *Cx. salinarius*) had taken bloodmeals from alligators in Louisiana (Chapter 3). The association of mosquitoes and alligators by bloodmeal identification is an important component of WNV vector incrimination. Another important element of vector incrimination is establishing spatial association of competent WNV mosquito vectors and captive alligator populations. Furthermore, establishing the temporal association of competent WNV mosquito vectors with the times of reported WNV outbreaks in captive alligators also is important (Eldridge and Edman 2000).

The purpose of this study was to establish spatial and temporal association of potential WNV vectors and captive alligators at two alligator farms where previous WNV outbreaks had occurred. This study was part of a comprehensive effort to investigate the potential role of mosquitoes for the introduction of WNV into captive alligator populations in Louisiana. A three year study was conducted to describe the composition of and WNV presence in mosquito populations inside and outside of alligator houses.

Materials and Methods

Experimental Design

Field studies were conducted at two alligator farms with previous history of WNV outbreaks in Louisiana. Alligator farm A was located in East Baton Rouge Parish, and alligator farm B was located in Terribonne Parish. Alligator farm A (30.370167 N, 90.975631 W) was located close to a suburban residence area with mixed vegetation intersected by several large ditches. Alligator farm B (29.5795N- 90.82505W) was surrounded by swampland.

Three Centers for Disease Control (CDC) light traps were placed at each alligator farm. One CDC light trap with an incandescent light and two CDC light traps with ultraviolet lights

were placed at alligator farm A. Three CDC light traps with ultraviolet lights were placed at alligator farm B. In addition to the CDC light traps, gravid traps (Reiter, 1983) baited with fish oil emulsion (Alaska Fish Fertilizer® Lilly Miller Brands, Clackamas, OR), backpack aspirators and resting boxes were used to collect mosquitoes. The CDC light traps were located both inside (one with ultraviolet light at each farm) and outside (two at each farm) the alligator houses at the alligator farms. Gravid traps and resting boxes were placed in areas with vegetation. The resting boxes were 30.5x30.5x30.5 cm wooden boxes, painted black on the outside and red on the inside. Backpack aspirators (Bioquip Products, Inc., Rancho Dominguez, CA) were used to collect mosquitoes from resting boxes and from vegetation. Traps were placed at the farms once per week in the afternoon and collections were made the following morning. Collections were made at alligator farm A from 28 July 2004 to 22 November 2005 and from 4 April 2006 to 9 June 2006. Collections were made at alligator farm B from 23 August 2005 to 25 October in 2005 and to 4 April 2006 to 14 November 2006.

Mosquito Processing

Mosquitoes were placed in containers marked with trap number, date, and location and transported to the laboratory in an ice chest containing dry ice. In the laboratory, mosquitoes were separated on a chill table (Industrial Inventions, Inc. West Windsor, NJ, USA) according to their species and placed into groups of bloodfed females, non-bloodfed females, and males. Non-bloodfed females and males were placed into cryovials in pools of 1-50 mosquitoes, stored at –80°C, and later tested for the presence of WNV. The heads and thoraxes of the bloodfed mosquitoes were removed placed into cryovials, stored at –80°C, and later tested for the presence of WNV. The abdomens of the bloodfed females were stored individually at –80°C until used for bloodmeal identification (Chapter 3).

Mosquito Pool Testing

All mosquito pools were tested at the Louisiana Animal Disease and Diagnostic Laboratory (LADDL) by RT-PCR for presence of WNV RNA. Mosquitoes were homogenized in 1 ml of BA-1 diluent with copper-coated steel beads (Lanciotti et al. 2000). Mosquitoes were homogenized using a TissueLyser (Qiagen, Valencia, CA, USA), for 4 min at 25 Hz, and then homogenates were centrifuged for 2 min at 5,796 x g. A volume of 220 µl of cleared homogenate was used for RNA extraction. RNA extraction was performed using Qiagen QIAamp® Virus Biorobot® 9604 Kit (Qiagen, Valencia, CA, USA). Briefly, homogenates were mixed with 240 µl AL buffer and 40 µl of protease, and then incubated at 60° C for 10 min. After the addition of 265 µl of 100% ethanol, samples were transferred to QIAamp® 96 plate (Qiagen, Valencia, CA, USA) and subject to three washes, RNA was eluted from the Qiagen columns in a volume of 86 µl elution buffer. Centrifugation at 5,796 x g was used to perform washes and elute. Elute was stored at -20° C until testing (Lanciotti et al. 2000; Eisler et al. 2004).

Real-time RT-PCR was performed by using QuantiTect Probe RT-PCR Kit (Qiagen, Valencia, CA, USA) with 15 pmol of each primer, 3 pmol of probe, and 5 µl of eluted RNA in a 15 µl total reaction volume. Primer sequence forward 5´TCAGCGATCTCTCCACCAAAG3´ and primer sequence reverse 5´GGGTCAGCACGTTTGTCATTG3´ were used to amplify the envelope gene (Lanciotti et al. 2000). The WNV RNA was detected as an increase in the fluorescence of the probe FAM-5´TGCCCGACCATGGGAGAAGCTC3´-BHQ1. The samples were subjected to 45 cycles of amplification in an ABI 7900HT real time PCR instrument (PE Applied Biosystems). The following cycling times and temperatures were used: 1 cycle of 48° C for 30 min and 95° C for 10 min followed by 45 cycles of 95° C for 15 sec, and 60° C for 1 min. Samples were interpreted as positive if the cycle threshold (CT) units were less than 40.

Statistical Analysis

Mosquito infection rates were determined by calculating the maximum likelihood estimate (MLE) with 95% confidence intervals using a computer based program (http://www.cdc.gov/ncidod/dvbid/westnile/software.htm).

Results

From 2004 to 2006, a total of 26,504 specimens (1,361 mosquito pools) representing 19 mosquito species, were collected at alligator farm A and tested for WNV (Table 4.1). Members of the genus *Culex* made up 84.2 % (n=22,332) of all mosquitoes captured over the 3-year period, and 62% of the *Culex* specimens were individuals of *Culex quinquefasciatus* Say. Mosquitoes of the following species also were collected and tested for WNV: *Aedes albopictus* (Skuse), *Ae. vexans* (Meigen), *Anopheles crucians* (Wiedemann), *An. punctipennis* (Say), *An. quadrimaculatus* Say, *Coquillettidia perturbans* (Walker), *Culiseta inornata* (Williston), *Cx. coronator* Dyar and Knab, *Cx. erraticus* (Dyar and Knab), *Cx. nigripalpus* Theobald, *Cx. restuans* Theobald, *Cx. salinarius* Coquillett, *Cx. tarsalis* Coquillett, *Mansonia titillans* (Walker), *Ps. ciliata* (Fabricius), *Ps. columbiae* (Dyar and Knab), *Ps. ferox* (Von Humboldt), and *Ps. howardii* Coquillett.

West Nile virus was detected in 28 pools of females of seven mosquito species (*Cx. coronator*, *Cx. quinquefasciatus*, *Cx. erraticus*, *Cx. nigripalpus*, *Ps. columbiae*, *An. quadrimaculatus*, and *Cq. perturbans*) at alligator farm A. We collected a total of 9,331 mosquitoes inside the alligator houses; WNV was detected in nine pools containing specimens of two mosquito species, (*Cx. quinquefasciatus* and *Cx. coronator*), and in one pool of *Cx. spp*. females. Of the six mosquito species we collected at alligator farm A, the MLE was calculated for species of which ≥ 1,000 individuals had been collected during one year. In 2004, the MLE

value of *Culex spp*. male mosquitoes (8.19/1,000) was 3.7 times greater than *Cx. quinquefasciatus* female MLE value (2.19/1,000); in 2005 MLE values were similar for *Cx. quinquefasciatus* females (1.68/1,000) and *Culex spp*. male (1.99/1,000) at the alligator farm A. In 2005, the MLE value for *Cx. nigripalpus* was 1.40/1,000 at alligator farm A.

From 2005 to 2006, a total of 32,664 specimens (1,043 mosquito pools) representing 19 mosquito species, were collected at alligator farm B and tested for WNV (Table 4.2). Members of the genus *Culex* made up 75.6 % (n=24,553) of all mosquitoes captured over the 2-year period, and 34% of the *Culex* specimens were individuals of *Cx. nigripalpus*. Mosquitoes of other species collected at alligator farms and tested for WNV were the same that were collected and tested from alligator farm A, excluding *An. punctipennis, Cx. coronator, Cx. tarsalis,* and *Px. ferox* but including *Ochlerotatus sollicitans* (Walker), *Oc. taeniorhynchus* (Weidemann), *Oc. infirmatus* Dyar and Knab, *Uranotaenia lowii* Theobald, and *Ur. sapphirina* (Osten Sacken).

West Nile virus was detected in 13 pools of females of 7 mosquito species (*An. crucians, Cx. erraticus, Cx. nigripalpus, Ma. titillans, Oc. sollicitans, Ps. columbiae, Ur. lowii*) at alligator farm B. We collected a total of 119 mosquitoes inside the alligator houses, and of those mosquitoes, WNV was detected in one pool containing specimens of *Cx. nigripalpus*. Of the five mosquito species we collected at alligator farm B, the MLE was calculated for species of which ≥ 1,000 individuals had been collected during one year. In 2005, none of the mosquito pools were positive for WNV RNA. In 2006, the MLE value for *Cx. erraticus* was 1.01/1,000, and the MLE value for *Cx. nigripalpus* was 0.12/1,000 at alligator farm B.

From 2004 to 2006, WNV was detected in seven species of mosquitoes at alligator farm A. Over the three year period, *Cx. quinquefasciatus* mosquitoes were collected in each month of

Table 4.1 Total number of mosquitoes collected from alligator farm A in East Baton Rouge Parish, 2004-2006

Species	Alligator Farm A											
	Gravid Trap			CDC Light Trap (inside)			CDC Light Trap (outside)			Aspiration+Resting box		
	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +
Ae. albopictus	52	4	0	36	8	0	23	9	0	1	1	0
Ae. vexans	15	8	0	8	3	0	3247	149	0	13	6	0
An. crucians	0	0	0	0	0	0	31	19	0	1	1	0
An. punctipennis	0	0	0	0	0	0	6	6	0	0	0	0
An. quadrimaculatus	1	1	0	1	1	0	60	19	3	16	11	2
Cq. perturbans	0	0	0	0	0	0	6	5	0	1	1	1
Cs. inornata	0	0	0	0	0	0	108	16	0	0	0	0
Cx. coronator	9	2	0	2	1	1	273	26	1	1	1	0
Cx. erraticus	4	2	0	2	2	0	147	26	1	29	12	1
Cx. nigripalus	15	5	0	6	4	0	1008	61	1	9	4	0
Cx. quinquefasciatus	3464	145	4	5777	136	8	3340	125	0	1250	107	1
Cx. restuans	233	20	0	2411	54	0	181	28	0	116	19	0
Cx. salinarius	0	0	0	2	1	0	234	24	0	2	2	0
Cx. spp (females)	119	14	0	210	18	1	17	2	0	34	3	0
Cx. spp (males)	747	40	0	873	37	0	161	7	2	1653	61	2
Cx. tarsalis	0	0	0	1	1	0	2	1	0	0	0	0
Ma. titillans	0	0	0	2	1	0	147	35	0	2	1	0
Ps. columbiae	6	1	0	0	0	0	347	45	1	0	0	0
Ps. ferox	0	0	0	0	0	0	38	10	0	0	0	0
Ps. ciliate	0	0	0	0	0	0	10	8	0	0	0	0
Ps. howardii	0	0	0	0	0	0	1	1	0	0	0	0

Table 4.2 Total number of mosquitoes collected from alligator farm B in Terribonne Parish, 2005-2006

Species	Alligator Farm B											
	Gravid Trap			CDC Light Trap (inside)			CDC Light Trap (outside)			Aspiration		
	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +
Ae. albopictus	0	0	0	0	0	0	2	1	0	7	3	0
Ae. vexans	0	0	0	0	0	0	375	23	0	2	1	0
An. crucians	10	3	0	2	1	0	1403	50	1	0	0	0
An. quadrimaculatus	2	2	0	26	5	0	465	37	0	2	2	0
Cq. perturbans	1	1	0	4	2	0	1835	55	0	0	0	0
Cs. inornata	1	1	0	0	0	0	0	0	0	0	0	0
Cx. erraticus	54	3	0	29	4	0	5465	135	6	47	4	0
Cx. nigripalus	8	3	0	28	4	1	8305	195	0	19	4	0
Cx. quinquefasciatus	1499	57	0	2	1	0	339	19	0	0	0	0
Cx. restuans	7	1	0	0	0	0	103	7	0	0	0	0
Cx. salinarius	31	6	0	14	1	0	1925	53	0	9	3	0
Cx. spp (females)	111	5	0	0	0	0	6183	143	0	0	0	0
Cx. spp (males)	329	21	0	0	0	0	39	6	0	7	2	0
Ma. titillans	0	0	0	14	4	0	2376	70	1	5	3	0
Oc. sollicitans	0	0	0	0	0	0	15	4	1	2	0	0
Oc. taeniorhynchus	0	0	0	0	0	0	8	4	0	0	0	0
Oc. infirmatus	0	0	0	0	0	0	137	11	0	8	3	0
Oc. spp. (females)	0	0	0	0	0	0	212	12	0	8	4	0
Ps. columbiae	0	0	0	0	0	0	346	16	1	0	0	0
Ps. ciliata	0	0	0	0	0	0	1	1	0	0	0	0
Ur. lowii	27	9	1	0	0	0	598	31	1	0	0	0
Ur. sapphirina	0	0	0	0	0	0	17	7	0	0	0	0

the year. West Nile virus positive *Cx. quinquefasciatus* mosquitoes were detected at alligator farm A between the months of July and September. Specimens of the *Cx. nigripalpus* mosquitoes were collected from May to November and WNV RNA was detected from *Cx. nigripalpus* mosquitoes during late September. Specimens of *Cx. coronator* mosquitoes were collected between the months of May and December, and WNV RNA was detected in one pool of *Cx. coronator* collected on 20 July. Specimens of *Cx. erraticus* mosquitoes were collected between the months of May and December and WNV RNA was detected in one pool of *Cx. erraticus* collected on 31 August. Specimens of *Cq. perturbans* mosquitoes were collected between the months of June and October and WNV RNA was detected in one pool of *Cq. perturbans* collected on 19 October. Over the three year period, specimens of *Ps. columbiae* mosquitoes were collected between the months of June and November and WNV RNA was detected in one pool of *Ps. columbiae* collected on 27 July. Specimens of *An. quadrimaculatus* mosquitoes were collected between the months of April and September and WNV virus were detected between the months of July and September.

From 2005 to 2006, WNV was detected in seven species of mosquitoes at alligator farm B. Specimens of *Culex erraticus* mosquitoes were collected between the months of May and October and WNV positive *Cx. erraticus* mosquitoes were detected between July and October. Specimens of *Cx. nigripalpus* mosquitoes were collected between August and October, and WNV was detected in *Cx. nigripalpus* mosquitoes during late September. Specimens of *An. crucians* mosquitoes were collected between the months of May and November, and WNV RNA was detected in one pool of *An. crucians* collected on 18 August. Specimens of *Oc. sollicitans* mosquitoes were collected between the months of September and October, and WNV RNA was detected in one pool of *Oc. sollicitans* collected on 4 September. Specimens of *Ma. titillans*

mosquitoes were collected between the months of August and November, and WNV RNA was detected in one pool of *Ma. titillans* collected on 17 October. Specimens of *Ps. columbiae* mosquitoes were collected between the months of July and October, and WNV RNA was detected in one pool of *Ps. columbiae* collected on 14 July. Specimens of *Ur. lowii* mosquitoes were collected between the months of May and November and WNV was detected in *Ur. lowii* mosquitoes collected during August.

Discussion

Mosquitoes of eleven species (*An. quadrimaculatus*, *An. crucians*, *Cq. perturbans*, *Cx. coronator*, *Cx. erraticus*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Ma. titillans*, *Oc. sollicitans*, *Ps. columbiae*, and *Ur. lowii*) were collected at the two alligator farms and tested positive for WNV RNA. Of those eleven species, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, and *Cx. quinquefasciatus* have been shown to feed on alligators (Rodrigues and Maruniak 2006, Chapter 3).

There have been no vector competence studies for eight (*An. quadrimaculatus*, *An. crucians*, *Cx. coronator*, *Cx. erraticus*, *Ma. titillans*, *Oc. sollicitans*, *Ps. columbiae*, and *Ur. lowii*) of the eleven mosquito species that were found to be positive for WNV RNA at the alligator farms. However, WNV has been detected repeatedly from specimens of each of these species, except *Ur. lowii* (CDC, 2007). Therefore, most of these species cannot be excluded as potential WNV vectors for captive alligators; particularly *Cx. erraticus* which has been shown to feed on alligators.

West Nile virus also was detected in *Cq. perturbans*, which has been described as an inefficient laboratory vector (Sardelis et al. 2001). However, WNV RNA has been detected in this species previously (Cupp et al. 2007), and Sardelis et al. (2001) considered *Cq. perturbans*

as a potential WNV vector due to its bird feeding behavior. Since *Cq. perturbans* was shown to feed on alligators (Chapter 3), the role of this species for WNV transmission in captive alligator farms should be investigated further.

Of the eleven mosquito species that were found to be positive for WNV RNA at the alligator farms, *Cx. quinquefasciatus* and *Cx. nigripalpus* have been identified as moderately competent vectors of WNV in laboratory studies (Sardelis et al. 2001). However, *Cx. quinquefasciatus* and *Cx. nigripalpus* both are considered to be primary enzootic and epidemic vectors of WNV in the southeastern U.S. (Godsey et al. 2005). We did collect specimens of the known competent vector *Cx. salinarius* (Turell et al. 2001) during the study, but we did not detect WNV RNA in these mosquitoes. However, we can not rule out the importance of *Cx. salinarius* in WNV transmission in captive alligators since female *Cx. salinarius* have been shown to feed on alligators (Chapter 3).

West Nile virus is primarily maintained in an enzootic transmission cycle between birds and mosquitoes. An effective vector to transmit WNV to captive alligators would be a mosquito species that feeds on birds but also opportunistically on other animals. All of the mosquito species found to be WNV positive at the alligator farms are considered to be opportunistic feeders, except *An. quadrimaculatus*, *Ps. columbiae* and *Oc. sollicitans* which feed primarily on mammals (Clements 1999).

Nevarez et al. (2005) reported WNV outbreaks at four alligator farms in Louisiana (including alligator farm A and B), and all cases were observed between August and December 2003 (J. Nevarez personal communication). The time between an alligator being infected with WNV by a mosquito bite and the determination of an outbreak in an alligator house (tankmates infected by contaminative transmission) would be at least 12-15 days (Klenk et al. 2004). In this

study, specimens of all of the mosquito species found WNV positive were collected and also found WNV positive between the months of July and October, which matches the timing of the reported WNV outbreaks at alligator farms in Louisiana.

West Nile virus outbreaks were reported from alligator farm A on 26 August 2003 and from alligator farm B on 16 October 2003 (J. Nevarez personal communication). At farm A, specimens of six of the seven species (excluding *Cq. perturbans*) shown to be WNV positive were collected and also found WNV positive between the months of July and August. At farm B, specimens of six of the seven species (excluding *Ps. columbiae*) shown to be WNV positive were collected and also found WNV positive between the months of September and October. Therefore, the timing of WNV positive mosquito pools detected at the two farms coincides with the reported WNV outbreaks.

Of the mosquitoes shown to be WNV positive collected from the farms at periods between July and November, four species (*Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, and *Cx. quinquefasciatus*) of those mosquitoes have been shown to feed on alligators between the months of March and November (Chapter 3). The temporal and spatial association of WNV RNA detection from specimens of these four species at alligator farms where WNV outbreaks occurred between August and October 2003, as well as the demonstration that these species feed on captive alligators suggest that these and possibly other mosquitoes may be a source of WNV outbreaks in captive alligators in Louisiana.

Barnett (1962) suggested four requirements for vector incrimination, and two of those requirements were addressed in Chapter 3. The third criterion for the incrimination of suspected arthropod as vectors requires repeated isolation or demonstration of the infection of the arthropod in nature. Mosquitoes of nine species were shown to be WNV positive between the

months of July and October which coincides with the reports of clinical infections in alligators. The fourth criterion, for vector incrimination would be the experimental transmission of WNV by mosquitoes to the alligators, which should be addressed in future studies.

The results of this study, strongly suggest that mosquitoes play an important role in WNV transmission for captive alligators. Generic adult mosquito control efforts at commercial alligator farms could be beneficial in reducing WNV transmission. Although WNV was detected in eleven species of mosquitoes in this study, the majority of the positive samples have been from *Cx. quinquefasciatus* and *Cx. nigripalpus* and both of these species were shown to feed on alligators. Therefore, larval control for these two species may be highly valuable in reducing WNV transmission in captive alligators. Optimal larval habitats of *Cx. quinquefasciatus* are mostly in stagnant water with heavy organic content. Large scale waste disposal from the alligator farms make these areas particularly attractive for mosquitoes. Efforts to eliminate *Cx. quinquefasciatus* larval habitats by mechanical methods could be recommended for alligator producers as well as application of larvicides where the larvae are present. The major larval habitats of *Cx. nigripalpus* are buckets, tires, fish ponds, rooting pails, and pools (Pirovost 1969) and these habitats could be eliminated or treated with larvicides to help reduce the potential of WNV transmission. Our study suggests that mosquito control should be considered to aid in the reduction of potential economical losses due to WNV related mortality and morbidity at alligator farms in Louisiana.

CHAPTER 5: VERTICAL TRANSMISSION OF WEST NILE VIRUS IN FIELD COLLECTED MOSQUITOES FROM EAST BATON ROUGE PARISH, LOUISIANA

Introduction

The first documented introduction of West Nile virus (WNV) into the United States was associated with human cases in New York City in 1999. From 1999 to 2002, the rapid spread of WNV to most regions of the continental United States was attributed to the movements of birds. The rapid spread and the establishment of WNV throughout most of North America indicated that WNV was transmitted by many different competent vectors, amplified in many different hosts, and had efficient mechanisms for maintenance within mosquito populations (Zeller and Schuffenecker 2004).

Birds are the primary reservoir hosts for WNV and the primary vectors are mosquitoes (Abramovitz 2004). The identity of the avian species that are the most important reservoirs for WNV in North America remains unclear (Komar 2003). During 2000, 3,687 dead birds, representing 153 species, 46 families, and 18 orders were tested for WNV, and there were 1,203 WNV-positive birds, representing 63 species, 30 families and 14 orders (Kramer and Bernard). However, surveillance data on avian deaths and seroprevalence studies do not indicate the competence of a particular species to infect mosquitoes. Komar et al. (2003) exposed 25 bird species to WNV and demonstrated that passerine birds, charadriiform birds, and at least two species of raptors (American Kestrel and Great Horned Owl) were more competent than species evaluated from the Anseriformes, Columbiformes, Galliformes, Gruiformes, Piciformes and Psittaciformes.

Since 1999, individuals from 62 mosquito species have been found to be WNV RNA positive in the United States. Depending upon environmental conditions, the average life span of the female mosquito is normally 3-4 weeks (CDC, 2004). Experimental infection studies have

shown that WNV infected birds, particularly passerines, are only infectious for mosquitoes for a period of 1-6 days (Komar et al. 2003). Although there are a large number of birds that are potential WNV amplification hosts and a large number of competent mosquito vectors of WNV, due to the short periods of viremia in birds and the short life span of mosquitoes, there are periods in each region of the range of WNV when active horizontal transmission does not occur. The mechanisms by which WNV persist through these periods is poorly described. For example, how WNV persists in cooler seasons when vectors are inactive and then reinitiates enzootic and/or epidemic transmission in the spring in the northeastern United States remains unclear (Anderson at al. 2006). Following periods without active horizontal transmission, other mechanisms could result in the reoccurence of WNV transmission: 1) reintroduction of the virus by migrating birds, 2) latent and recrudescence of infections in birds, 3) horizontally WNV infected, diapausing female mosquitoes, or 4) vertical transmission.

There is little doubt that introduction of WNV by birds into areas devoid of horizontal transmission between birds and mosquitoes does occur. West Nile virus has been isolated from migrating birds including, Barred Warblers in Cyprus and Turtle Doves in Slovakia (Rappole et al. 2000). Malkinson et al. (2002) isolated WNV from White Storks that were grounded in Israel while migrating southward on a route that took them along the eastern edge of the Syrian-African Rift Valley, Jordan. The authors found WNV positive fledglings that had hatched in Europe in the spring of the same year and had yet to complete a full migratory cycle to Africa. Reisen et al. (2001) investigated the hypothesis that chronic infections of WNV could be established in resident bird species, persist during cooler seasons, and then relapse during the next transmission season. However, the results of their study did not support the recrudescence of virus either in resident or migrating birds.

The two ways that have been proposed by which WNV can persist in mosquito populations during periods absent of horizontal transmission are: 1) survival of female mosquitoes infected by horizontal transmission and then entering diapause, and 2) vertical transmission. Although gonotrophic dissociation in diapausing females that were horizontally infected has been proposed to explain persistence of WNV during the absence of horizontal transmission (overwintering), gonotrophic dissociation has not been observed directly in natural populations of *Culex* mosquitoes (Mitchell and Briegel 1989). Since almost all overwintering females of Culex species females are nulliparous, overwintering females are likely infected with WNV via direct vertical transmission or venereally from a male that was infected via vertical transmission. The importance of venereal transmission of WNV for eventual horizontal transmission is unclear. Reisen et al. (2006) demonstrated that *Culex* females can be infected venereally, however those females did not amplify virus after mating and the single female progeny of that retained WNV for 3 d were negative for WNV.

There are several recent studies that provided evidence of vertical transmission of WNV in nature. Miller at al. (2000) first reported vertical transmission for WNV in nature from *Cx. univittatus* (Theobald) males collected from Rift Valley Province of Kenya. Reisen et al. (2006) demonstrated vertical transmission of WNV in *Cx. pipiens* complex by testing field collected male mosquitoes and larvae in California. Phillips and Christensen (2006) detected WNV from field collected 3rd and 4th stage *Cx. erythrothorax* Dyar larvae in Utah.

Anderson et al. (2006) reported that a vertically infected female *Cx. pipiens* fed on a hamster that died 8 days later of WNV infection. Therefore, female mosquitoes infected with WNV by vertical transmission and entering diapause can initiate horizontal transmission the following spring. Vertical transmission in mosquitoes most likely contributes to the maintenance

of WNV in nature in temperate climates. In the studies of Chapter one and Chapter three, male mosquitoes were collected and found to be WNV positive. The purpose of this study was to conduct a more comprehensive investigation of vertical transmission of WNV in mosquitoes.

Materials and Methods

Adult Mosquito Sampling

Male mosquitoes were collected at the sites described in Chapter one and there were two urban locations (Site I and Site II) and two suburban locations (Site III and Site IV). Site I (30.49223N-91.16052W) was an open location with a very little vegetation. Site II (30.45578N-91.00609W) was an open area with mixed vegetation. Site III (30.45578N- 91.12119W) was a small farm, and Site IV (30.57262N-91.07115W) was a wooded area intersected by several large ditches. In 2005 and 2006, Site I (30.38097N-91.20696W) was a horse activity center with wooded areas and open pasture, and Site II (30.56209N-9111533W) was an urban residence with mixed vegetation, while sites III and IV remained the same. Mosquitoes were collected using a backpack aspirator from vegetation, sentinel chicken boxes, and resting boxes (Bioquip Products, Inc. Rancho Dominguez, CA, USA). From 28 March 2006 to 16 October 2006, we also processed field collected male mosquitoes from 21 sites which were selected by East Baton Rouge Mosquito and Rodent Control (EBRMARC) personnel to represent a diversity of habitats (urban areas, suburban areas, parks, and agricultural land), and also based on past WNV activity (Table 4.1). Male mosquitoes were collected once per week by EBRMARC inspectors by using Centers for Disease Control (CDC) light traps and gravid traps (Alaska Fish Fertilizer® Lilly Miller Brands, Clackamas, OR). All mosquitoes were placed into cryovials in pools of 1-50 mosquitoes and stored at –80° C and subsequently tested for the presence of WNV RNA.

Larvae Sampling

Mosquito larvae were collected once per week by EBRMARC inspectors from six sites out of 43 different sites, located throughout East Baton Rouge (EBR) Parish. Four weekly collection sites were selected from areas where WNV was detected in female mosquitoes. Gravid trap pans baited with fish oil emulsion were placed at selected sites and larval collections were made 5 days later. Two of the sites were Site IV of Chapter one (30.57262N-91.07115W) and alligator farm A of Chapter two (30.370167N, 90.975631W) and dippers were used to collect larvae from larval habitats. The larvae collected at each site were combined and placed in pans in separate cages with date and location labels. Larvae were held in the EBRMARC insectary at 28°C and 50-70% RH.

After emergence, mosquitoes were aspirated from the cages using a backpack aspirator (Bioquip Products, Inc., Rancho Dominguez, CA). The mosquitoes were held in a freezer at –20° C until dead, and then separated by species and sex on a chill table (Industrial Inventions, Inc. West Windsor, NJ, USA). Both female and male mosquito pools were tested at Louisiana Animal Disease and Diagnostic Laboratory (LADDL) by Real-time Reverse-Transcription PCR (RT-PCR) for presence of arbovirus RNA.

Mosquito Pool Testing

All mosquito pools were tested at the LADDL by RT-PCR for presence of WNV RNA. Mosquitoes were homogenized in 1 ml of BA-1 diluent with copper-coated steel beads (Lanciotti et al. 2000). Mosquitoes were homogenized using a TissueLyser (Qiagen, Valencia, CA, USA), for 4 min at 25 Hz, and then homogenates were centrifuged for 2 min at 5,796 x g. A volume of 220 µl of cleared homogenate was used for RNA extraction. RNA extraction was performed

Table 5.1 Locations of the trap sites for adult male mosquito collection in East Baton Rouge Parish, Louisiana

Site	Latitude	Longitude	Description of Site
Keokuk Street	30.48737N	-91.15911W	Residential Area
Drusilla Lane	30.41323N	-91.09011W	Residential Area
Duchess Park	30.28092N	-91.02264W	Residential Park
Castle Ridge Avenue	30.35917N	-91.01340W	Residential Area
McGraw Lane	30.44236N	-90.96763W	Residential Area
Marilyn Drive	30.26348N	-91.04541W	Residential Area
Mills Avenue	30.32160N	-91.12069W	Levee area next to residential
Morgan Road	30.30412N	-90.59418W	Residential
Red Oak Drive	30.27441N	-91.03565W	Residential next to large wooded area
Stoney Point Burch Road	30.39008N	-90.57404W	Rural-Residential
Lemon Road	30.71558N	-91.14256W	Rural Area
Peairs	30.63883N	-91.14140W	Rural residential
City Park	30.4328N	-91.17032W	Residential Park
Highland Road	30.34955N	-91.06788W	Residential Area
Lee Drive High School	30.40415N	-91.15134W	School Area
Farr Park	30.38559N	-91.20426W	Horse Stable
Pecue Lane	30.38165N	-91.04576W	Residential Area
O'Neal Lane	30.43324N	-91.00738W	Commercial Area
Greenwell Springs Road	30.49306N	-91.08361W	Commercial Area
Denham Road	30.59223N	-91.04027W	Residential Area
Greenwood Park	30.57028N	-91.17250W	Residential Park

using Qiagen QIAamp® Virus Biorobot® 9604 Kit (Qiagen, Valencia, CA, USA). Briefly, homogenates were mixed with 240 µl AL buffer and 40 µl of protease, and then incubated at 60° C for 10 min. After the addition of 265 µl of 100% ethanol, samples were transferred to QIAamp® 96 plate (Qiagen, Valencia, CA, USA) and subject to three washes, RNA was eluted from the Qiagen columns in a volume of 86 µl elution buffer. Centrifugation at 5,796 x g was used to perform washes and elute. Elute was stored at -20° C until testing (Lanciotti et al. 2000; Eisler et al. 2004).

Real-time RT-PCR was performed by using QuantiTect Probe RT-PCR Kit (Qiagen, Valencia, CA, USA) with 15 pmol of each primer, 3 pmol of probe, and 5 µl of eluted RNA in a 15 µl total reaction volume. Primer sequence forward 5´TCAGCGATCTCTCCACCAAAG3´ and primer sequence reverse 5´GGGTCAGCACGTTTGTCATTG3´ were used to amplify the envelope gene (Lanciotti et al. 2000). The WNV RNA was detected as an increase in the fluorescence of the probe FAM-5´TGCCCGACCATGGGAGAAGCTC3´-BHQ1. The samples were subjected to 45 cycles of amplification in an ABI 7900HT real time PCR instrument (PE Applied Biosystems). The following cycling times and temperatures were used: 1 cycle of 48° C for 30 min and 95° C for 10 min followed by 45 cycles of 95° C for 15 sec, and 60° C for 1 min. Samples were interpreted as positive if the cycle threshold (CT) units were less than 40.

Standard Curve for RT-PCR

A standard curve was generated using five replicates of a standard curve created with 10-fold serial dilutions of RNA extracted from virus stock with a known titer of 1.77x107 PFU/mL. Standard curves included laboratory reared *Culex quinquefasciatus* Say mosquitoes (Sebring strain) in addition to the known virus concentrations. Three standard curves consisted of 5, 25 or 50 mosquitoes per tube spiked with known concentrations of virus. Samples were analyzed in comparison to appropriately matched standard curves. The quantity of virus per sample was determined by using the ABI software (PE Applied Biosystems).

Statistical Analysis

Mosquito infection rates were determined by calculating the maximum likelihood estimate (MLE) with 95% confidence intervals using a computer based program (http://www.cdc.gov/ncidod/dvbid/westnile/software.htm).

Results

From 2004 to 2006, a total 12,206 adult male mosquitoes were collected and tested for WNV RNA. The sampled species included *Aedes albopictus* (Skuse), *Ae. vexans* (Meigen), *Anopheles quadrimaculatus* Say, *Coquillettidia perturbans* (Walker), *Culiseta inornata* (Williston), *Cx. coronator* Dyar and Knab, *Mansonia titillans* (Walker), *Psorophora* ciliata (Fabricius), *Ps. columbiae* (Dyar and Knab), *Ps. howardii* Coquillett, *Uranotaenia lowii* Theobald, and *Ur. sapphirina* (Osten Sacken). Males of *Culex* species made up 91.7% of all the mosquitoes tested. West Nile virus was detected in 15 pools of male Culex specimens (Table 4.2). WNV positive males were captured between 7 May and 2 August in 2004. In 2005, WNV positive males were collected between 6 January and 10 November. Infected males were captured between 15 June and 19 September in 2006.

Of the mosquito larvae that were collected between 10 August and 30 November 2005 and between 27 March and 11 December 2006, 47,005 emerging adults were tested in 1,058 pools. West Nile virus was detected in two pools of *Cx. quinquefasciatus* females, one pool of *Ae. albopictus* females and two pools of *Culex spp*. males. Out of four WNV positive pools containing female or male *Culex* mosquitoes, mosquitoes of one female pool and one male pool were from the same collection. All of the females that emerged from the two larval samples that contained males positive for WNV were identified as *Cx. quinquefasciatus*.

Cycle threshold values were linear across 4 logs of viral RNA concentration, with correlation coefficients of 0.9983, 09968, and 0.9822. The virus concentration ranged from 6.46 x101 PFU / 220 µl to 3.85x103 PFU / 220 µl for pools of male mosquitoes collected during 2006 (Table 2). In 2006, at alligator farm A, the MLE value for *Cx. quinquefasciatus* females (collected as larvae) was 0.33/1,000, and the MLE value for male *Cx. spp*. was 0.29/1,000.

Discussion

In this study, WNV was detected from *Culex* males and nulliparous *Cx. quinquefasciatus* females. These findings represent the second report of WNV vertical transmission in *Cx. quinquefasciatus* in nature. Reisen et al. (2006) found that field collected males and immatures of *Cx. quinquefasciatus* were infected with WNV in California. Vertical transmission of WNV also has been reported from other field collected mosquitoes of three other *Culex* species; *Cx. univittatus* (Miller at al. 2000), *Cx. pipiens* complex (Reisen et al. 2006), and *Cx. erythrothorax* Phillips and Christensen (2006).

This is the first report of detection of WNV RNA from field collected nulliparous *Ae. albopictus* females. Baqar et al. (1993) demonstrated that WNV intrathoracically infected *Ae. albopictus* females had infected progeny. On the other hand, Dohm et al. (2002) tested more than 13,000 progeny of WNV intrathoracically infected *Ae. albopictus* but did not observe vertical transmission.

The efficiency of vertical transmission of WNV in *Culex* mosquitoes has been reported from laboratory studies. Reisen et al. (2006) infected *Cx. tarsalis* Coquillett females by intrathoracic inoculation and reported that five percent of the F1 progeny females were WNV positive. Mishra and Mourya (2001) found a similar result (5.56 % infected F1 progeny) with *Cx. vishnui* subgroup. In the present study, 626 *Culex* pools of mosquitoes (collected as larvae) were tested for WNV and 0.63% (4/626) of the pools were found positive for WNV RNA. During the same study period we processed 12 pools of *Ae. albopictus* mosquitoes and one pool of

Table 5.2 Adult male mosquitoes collected from EBR Parish, Louisiana, (2005-2006) and tested for WNV RNA by RT-PCR

Species	Gravid Trap			Sentinel Chicken Box Trap			CDC Light Trap			Aspiration+Resting box		
	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +	No mosq	No Pools Tested	No Pools WNV +
Ae. albopictus	47	5	0	80	5	0	30	8	0	4	1	0
Ae. vexans	0	0	0	2	1	0	9	4	0	0	0	0
An. quadrimaculatus	12	3	0	48	7	0	19	3	0	3	3	0
Cq. perturbans	0	0	0	40	2	0	1	1	0	0	0	0
Cs. inornata	0	0	0	10	2	0	1	1	0	0	0	0
Cx. coronator	0	0	0	0	0	0	13	1	0	0	0	0
Cx. spp	5013	232	4	2010	152	5	1566	78	3	2615	101	3
Ma. titillans	0	0	0	0	0	0	2	1	0	0	0	0
Ps. ciliata	0	0	0	0	0	0	0	0	0	0	0	0
Ps. columbiae	6	1	0	0	0	0	25	1	0	0	0	0
Ps. howardii	0	0	0	0	0	0	12	3	0	0	0	0
Ur. lowii	1	1	0	0	0	0	0	0	0	0	0	0
Ur. sapphirina	0	0	0	0	0	0	4	1	0	0	0	0

Table 5.3 Adult male mosquitoes and larvae collected from EBR Parish, Louisiana (27 March-11 December 2006) and tested for WNV RNA by RT-PCR

Mosquito species	Collection date	CT value	Pool size	WNV con. (PFU/220µl)
Culex spp. males [a]	05/30/06	38.0	50	$<4x10^{4}$ [c]
Culex spp. males [a]	06/21/06	34.4	9	$3.85x10^{3}$
Culex spp. males [a]	06/23/06	35.8	8	$1.48x10^{2}$
Culex spp. males [a]	08/04/06	36.2	11	$3.10\ x10^{2}$
Culex spp. males [a]	09/01/04	37.8	29	$1.60x10^{2}$
Ae. albopictus[b] females	08/01/06	38.2	2	$6.46\ x10^{1}$
Cx. quinquefasciatus[b] females	09/05/06	39.8	50	$<4x10^{4}$ [c]
Culex spp. males [b]	09/05/06	38.9	50	$<4x10^{4}$ [c]
Cx. quinquefasciatus[b] females	09/11/06	38.8	50	$<4x10^{4}$ [c]
Culex spp. males [b]	09/19/06	34.4	2	$2.79x10^{2}$

[a] Specimens collected as adults and tested for WNV RNA
[b] Specimens collected as larvae and reared in the laboratory and subsequently tested for WNV RNA
[c] Standard curves containing 50 mosquitoes did not provide enough points to calculate WNV concentration

nulliparous female mosquitoes was found positive for WNV RNA. In Chapter 2 and Chapter 4, MLE values for male mosquitoes ranged from 1.61/1,000 to 8.19/1,000, while for larval collection in this study the values ranged from 0.33/1,000 to 0.29/1,000. Anderson et al. 2006 found that 10% of WNV positive female mosquitoes (*Cx. pipiens*) were able to transmit the virus transovarially to 2.5% of their progeny. Therefore, the results of the studies in this dissertation indicated that the rate of vertical transmission of WNV in nature is close to that predicted in laboratory studies. In this study, WNV positive male mosquitoes were collected between the months of May and January, which also indicates that vertical transmission is not confined temporally.

The significance of vertical transmission of WNV in nature has not been established. However, vertical transmission followed by horizontal transmission could play an important role

for starting the transmission cycle in spring. Anderson et al. (2006) reported that one vertically infected female *Cx. pipiens* (progeny of a WNV infected female collected from the field during September) fed on a hamster that died 8 days later of WNV infection, which demonstrates that female mosquitoes infected with WNV by vertical transmission can be competent vectors. Although *Cx. quinquefasciatus* has been identified as moderately competent vector WNV (Sardelis et al. 2001) this species is considered to be the primary vector of WNV in the southeastern U.S. The importance of vertical transmission of WNV in the significant role that *Cx. quinquefasciatus* mosquitoes serve as enzootic and epizootic vectors of WNV may be of a higher magnitude than previously estimated.

The results of this study indicate that vertical transmission in certain mosquitoes does occur in Louisiana at many times of the year. Since vertically infected mosquitoes can enter diapause and then transmit WNV horizontally after diapause, overwintering of WNV in vertically infected female mosquitoes is likely to occur in northern climates. In Louisiana, vertical transmission also may be important for long-term maintenance of WNV in mosquito populations when there is no active horizontal transmission. The finding of vertical transmission in *Ae. albopictus* (field-collected) could have broad implications; this is the first report of vertical transmission in mosquitoes which have eggs that withstand long periods of desiccation.

SUMMARY AND CONCLUSIONS

Following its introduction in 1999, West Nile virus (WNV) has caused repeated large-scale human epidemics in North America and is now the dominant vector-borne disease in this continent. West Nile virus has caused over 25,000 reported human cases, 1,000 deaths during 1999-2007 in the United States. Although there are other mosquito-borne viral infections that occur in the United States including St. Louis encephalitis, Eastern and Western equine encephalitis, and LaCrosse encephalitis, none of them have caused human illness of the magnitude of WNV. Since there is no WNV specific treatment or vaccine available, the prevention of human disease is strongly based on effective surveillance programs, sustained mosquito control, and public education.

For the first part of this dissertation, we conducted a study to determine if testing mosquitoes collected in modified sentinel chicken boxes for West Nile Virus (WNV) or testing sentinel chickens for WNV antibody would detect WNV activity prior to reports of human cases in East Baton Rouge (EBR) Parish, LA. In each year, mosquitoes tested positive for WNV before human cases were reported, but seroconversions of sentinel chickens were detected after the onset of human cases. These results are consistent with findings in St. Tammany Parish, LA (Palmisano et al. 2005) which also reported that seronversions of sentinel chickens peaked at about the same time as human cases. In one year we also compared the effectiveness of CDC light traps, gravid traps, and sentinel chicken box traps for collecting WNV positive mosquitoes. In total, 1,222 pools containing 19,353 mosquito specimens collected between 2004-2006, representing 18 species, were analyzed for the presence of WNV RNA using reverse transcription polymerase chain reaction (RT-PCR). Gravid traps collected more WNV infected mosquitoes than CDC light traps or sentinel chicken box traps in the trap comparison. However,

WNV was detected earlier in mosquitoes collected from sentinel chicken box traps than in mosquitoes collected with gravid traps or CDC light traps. The result of this study suggest that testing mosquitoes collected in sentinel chicken box traps may be the best early predictor of human WNV cases in EBR Parish.

West Nile virus was first reported to infect American alligators (*Alligator mississippiensis*) in the United States in 2001, when WNV-associated deaths occurred in 250 alligators in commercial alligator houses in Georgia (Miller et al. 2003). In 2003, more than 700 WNV-associated hatchling deaths occurred at three Louisiana alligator farms (ProMed-mail, 2003). In the second part of this dissertation, we were able to fulfill three requirements for vector incrimination of mosquitoes as vectors of WNV for captive alligators. Mosquitoes were collected using Centers for Disease Control (CDC) light traps, gravid traps, backpack aspirators and resting boxes at three commercial Louisiana alligator farms from 2004 to 2006. The bloodmeal origins of 237 field-collected mosquitoes were identified based on cytochrome B sequence homology. Alligator blood was detected in 24 mosquitoes representing six species of mosquitoes, including *Ae. vexans*, *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, *Cx. quinquefasciatus*, and *Cx. salinarius*, and many of these species are considered to be competent vectors of WNV. Rodrigues and Maruniak (2006) showed that mosquitoes of three species (*Mansonia dyari*, *Ma. titillans*, and *Cx. erraticus*) had fed on captive alligators in Florida. However, none of these mosquito species are considered to be primary vectors of WNV in Florida. We also tested the heads and the thoraxes of bloodfed females, non-bloodfed females, and males for WNV RNA using RT-PCR. We collected and tested a total of 59,168 mosquitoes representing 24 species and WNV was detected in 41 pools of females of 11 mosquito species: *Anopheles crucians*, *An. quadrimaculatus*, *Coquillettidia perturbans*, *Culex coronator*, *Cx.*

erraticus, *Cx. nigripalpus*, *Cx. quinquefasciatus*, *Ma. titillans*, *Psorphora columbiae*, *Oc. sollicitans*, and *Uranotaenia lowii*. Specimens of all of the mosquito species found WNV positive were collected and also found WNV positive between the months of July and October, which matches the timing of the reported WNV outbreaks at alligator farms which occurred between August and October 2003. The temporal and spatial association of WNV RNA detection and alligator blood identification from *Cq. perturbans*, *Cx. erraticus*, *Cx. nigripalpus*, and *Cx. quinquefasciatus* with reported outbreaks of WNV in captive alligators suggests that mosquitoes may be a source of WNV infection of captive alligators in Louisiana.

In the last chapter of this dissertation, the occurrence of vertical transnsmission in EBR Parish in Louisiana in *Culex quinquefasciatus* and *Ae. albopictus* mosquitoes was demonstrated. Adult male mosquitoes were collected using Centers for Disease Control (CDC) light traps, gravid traps, backpack aspirators, resting boxes, and tested positive for WNV RNA by RT-PCR. From 2004 to 2006, a total 12,206 adult male mosquitoes were collected. Male mosquitoes of 12 species (*Aedes albopictus*, *Ae. vexans*, *An. quadrimaculatus*, *Culiseta inornata*, *Cx. coronator*, *Ma. titillans*, *Psorophora ciliata*, *Ps. columbiae*, *Ps. howardii*, *Ur. lowii*, and *Ur. sapphirina*) were collected and tested for WNV. West Nile virus RNA was detected in 15 pools of male *Culex* species. Mosquito larvae were collected using gravid trap pans and from mosquito larval habitats and 47,005 emerging adults were tested for WNV RNA from 2005 to 2006. West Nile virus was detected in 2 pools of *Cx. quinquefasciatus* nulliparous females, 1 pool of *Ae. albopictus* nulliparous females, and 2 pools of *Culex spp*. males. These findings represent only the second report of WNV vertical transmission in nature from *Cx. quinquefasciatus* and the first study that detected WNV from field collected nulliparous *Ae. albopictus* females. This is the first

report of vertical transmission in mosquitoes which have eggs that withstand long periods of desiccation.

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