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Detection of West Nile virus activity in male and female mosquitoes, and evaluation of host-utilization patterns of mosquitoes, in East Baton Rouge Parish, Louisiana

Andrew James Mackay
Louisiana State University and Agricultural and Mechanical College, amackay@agctr.lsu.edu

DETECTION OF WEST NILE VIRUS ACTIVITY IN MALE AND FEMALE MOSQUITOES, AND EVALUATION OF HOST-UTILIZATION PATTERNS OF MOSQUITOES, IN EAST BATON ROUGE PARISH, LOUISIANA

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Andrew J. Mackay
B.S.A., University of Manitoba, 1992
M.Sc., University of Manitoba, 1996
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DEDICATION

This dissertation is dedicated to my parents, Margaret and Jim Mackay, and my sisters Maureen DiBernardo and Elizabeth Miller. Their love and support made all things possible.

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ABSTRACT

Host utilization patterns of female mosquitoes, and WNV infection rates in male and female mosquitoes, were examined in East Baton Rouge Parish to identify potential vectors of West Nile virus (WNV). A total of 244,374 female mosquitoes were collected between November 2002 and October 2004 and tested by viral isolation. Additionally, 131,896 female mosquitoes were tested by VecTest in 2003, and 167,175 female mosquitoes were tested by RT-PCR in 2004. West Nile virus was isolated from 17 of 36 mosquito species assayed by virus isolation, WNV antigens were detected in 9 of 27 species tested by VecTest, and WNV RNA was detected in 14 of 28 mosquito species tested by RT-PCR. The species with the greatest number of WNV positive pools by all 3 testing methods was *Culex quinquefasciatus*. The Terminal Restriction Fragment Length Polymorphism assay and direct sequencing were used to determine the host bloodmeal identity in 37 bloodfed *Cx. coronator*, 67 bloodfed *Cx. salinarius*, 114 bloodfed *Cx. nigripalpus*, and 686 bloodfed *Cx. quinquefasciatus*. The proportions of bloodmeals containing mammalian DNA were 94.6% for *Cx. coronator*, 82.1% for *Cx. salinarius*, 66.1% for *Cx. nigripalpus*, and 40.1% for *Cx. quinquefasciatus*. The White-tailed Deer was the most common host of *Cx. coronator* and *Cx. salinarius*. The most common mammalian host of *Cx. nigripalpus* and *Cx. quinquefasciatus* was the Northern Raccoon. Human DNA was detected in 7% of the bloodmeals of *Cx. quinquefasciatus* and 2.7% of the bloodmeals of *Cx. nigripalpus*. The Northern Cardinal was the most frequent avian host of *Cx. quinquefasciatus* and *Cx. nigripalpus*. There was no seasonal change in the monthly proportion of females of *Cx. nigripalpus* and *Cx. quinquefasciatus* feeding on mammals from May through October, in either 2003 or 2004. A total of 45 pools containing 171 males of 12 species were assayed for the presence of WNV by virus isolation, and 148 pools containing 7230 males of 15

mosquito species were tested by RT-PCR. Virus was isolated from single pools of male *Cx. salinarius* and *Oc. triseriatus*. Single pools containing male *Cx. restuans, Ps. howardii, Oc. triseriatus* and *An. crucians* were positive for WNV RNA.

INTRODUCTION

In 1999, West Nile virus (WNV) was first detected in the western hemisphere. Within a few years, WNV transmission was detected throughout most of the continental United States and southern Canada, and several locations in Mexico, Central America and the Caribbean. Although considerable resources were dedicated to the study of WNV epizootiology in the northeastern United States, little was known of the transmission cycle in the Gulf Coast region.

In much of the southeastern United States, the mosquito species of greatest public health concern in urban areas is the Southern house mosquito, *Culex quinquefasciatus* Say. In this region, *Cx. quinquefasciatus* has been implicated as the primary enzootic vector of WNV and St. Louis Encephalitis virus (SLEV). However, evidence of WNV infection has been reported in at least 34 additional mosquito species known to occur in East Baton Rouge Parish (EBRP), Louisiana (Chapman and Johnson 1986, CDC 2007e). Of these 34 species, at least 5 are abundant in EBRP and have been demonstrated to be moderately or highly competent vectors of WNV in the laboratory. Knowledge of the seasonal dynamics of virus infection and host utilization in these species is required to assess their significance as enzootic and/or epidemic vectors of WNV in EBRP.

Since WNV was first detected in Louisiana in 2001, more than 900 human cases have been diagnosed; 60 of these cases were fatal. Reducing the risk of human exposure to WNV requires a targeted vector control program based on a clear understanding of the species involved in virus transmission. The research presented in this dissertation was conducted with the intent of increasing our comprehension of the role of different mosquito species in the transmission of WNV. West Nile virus infection rates were assessed in female and male mosquitoes to determine the frequency of horizontal and vertical transmission in different species. Host feeding patterns

also were evaluated in known or suspected mosquito vectors in an effort to clarify their role in the epizootiology of WNV in EBRP.

CHAPTER 1. LITERATURE REVIEW

1.1 WNV Virology and Taxonomy

West Nile virus (WNV) is a single stranded, positive-sense RNA virus in the genus *Flavivirus*, family Flaviviridae. The genome contains a single open reading frame of 10,301 nucleotides coding for three structural proteins (capsid, membrane/premembrane and envelope) and seven non-structural proteins (Rice *et al.* 1985, Lanciotti *et al.* 1999).

Flaviviruses are a highly genetically diverse group, with phylogeny strongly correlating with mode of transmission and host range (Gaunt *et al.* 2001, Kuno 2007). The genus includes viruses transmitted by mosquitoes, viruses transmitted by ticks, and viruses for which no vector is known (Calisher and Karabatsos 1988). Most mosquito-borne *Flavivirus* can be divided into two broad epidemiological groups, although phylogenetic relationships within and between the two groups have not been fully resolved (Cook and Holmes 2006). One group includes viruses primarily transmitted by *Aedes* species, maintained in mammalian reservoirs, and generally less neurotropic in humans and livestock; examples of this group include Dengue virus and Yellow Fever virus. The second group includes viruses primarily transmitted by *Culex* species, maintained in avian reservoirs, and sometimes associated with encephalitis in humans (Gaunt *et al.* 2001). West Nile virus is included in the second group.

West Nile virus is most closely related to members of the Japanese Encephalitis virus (JEV) antigenic complex, which includes Usutu virus, Cacipacore virus, Koutango virus, Yaounde virus, Japanese Encephalitis virus (JEV), Murray Valley encephalitis virus and St. Louis Encephalitis virus (SLEV) (Calisher *et al.* 1989, Heinz *et al.* 2000). There are at least 2 distinct lineages of WNV described, based on sequence homology of the gene coding for the envelope protein (Berthet *et al.* 1997). Lineage 1 has been further subdivided into at least 3 clades,

including a clade composed of strains from Europe, Africa, the Middle East and the Western Hemisphere, a clade represented by strains from India, and a clade containing strains of Kunjin virus, a subtype of WNV from Australia (Heinz *et al.* 2000, Lanciotti *et al.* 2002). Lineage 2 strains have only been isolated from sub-Saharan Africa and Madagascar, and have not been associated with human disease (Brinton 2002). Bakonyi *et al.* (2005, 2006) have proposed the recognition of 2 additional WNV lineages based on molecular characterizations of viruses collected from the Czech Republic and the Caucasus.

West Nile virus, like all RNA viruses, can undergo very rapid mutation (Holland *et al.* 1982). The first fully sequenced WNV strain from North America was isolated from a Chilean flamingo and designated NY99 (Lanciotti *et al.* 1999). Comparisons between NY99, and other isolates of WNV collected from other locations in North America, showed that the strains circulating in North America in 1999 and 2000 were relatively homogeneous (>99.8% homology) (Lanciotti *et al.* 1999, Lanciotti *et al.* 2002). Studies comparing sequence homology of North American strains circulating in 2001 and 2002 identified a small degree of nucleotide variability among strains (Beasley *et al.* 2003, Davis *et al.* 2003). By 2003, a new genotype (WN02), exhibiting a mutation in the envelope protein gene, was found to have become the dominant WNV strain throughout most of North America, displacing the NY99 genotype (Ebel *et al.* 2004, Davis *et al.* 2005, Herring *et al.* 2007). The extrinsic incubation period of WN02 has been shown to be shorter than NY99, possibly conferring a selection bias to WN02 (Ebel *et al.* 2004, Snapinn *et al.* 2007). Other distinct phenotypes identified in North America include isolates collected from Texas and Mexico in 2003 that demonstrate reduced neuroinvasiveness in mice (Beasley *et al* 2004, Davis *et al.* 2004).

1.2 Origin, Introduction and Establishment of WNV in the New World

The first isolation of WNV was from a woman in Uganda in 1937 (Smithburn *et al*. 1940). The first WNV epidemics were reported in Israel in the early 1950's (Hayes 1989). Throughout the latter half of the 20th century, sporadic outbreaks of human infection, typically a mild febrile illness without severe neuropathology, were reported in Africa, Europe, Asia, Australia and the Mediterranean region. The largest outbreak of WNV on record was in South Africa in 1974, with almost ten thousand human cases reported (McIntosh *et al.* 1976).

In 1996, the first important WNV outbreak in Europe occurred in Romania, with almost 400 confirmed human cases of WNV and 17 deaths (Tsai *et al.* 1998). This event also represented the first predominantly urban outbreak of WNV, and the first major outbreak where most of the symptomatic cases reported presented with acute central nervous system (CNS) infection. From 1996 to 1998, significant WNV activity also was reported in the Czech Republic, Poland, Italy, Russia, Israel, Morocco, Tunisia, Democratic Republic of Congo, Kenya, and Australia (Hubálek and Halouzka 1999, Murgue *et al.* 2002). The viral strains active in Israel were observed to be very pathogenic to domestic geese and wild birds (Bin *et al.* 2001, Malkinson and Banet 2002).

In 1999, WNV was first detected in the Western Hemisphere, in New York City (NYC) (Nash *et al.* 2001). Similar to the 1996 outbreak in Romania, most of the symptomatic cases of WNV in Russia and New York in 1999, and Israel in 2000, were from urban areas and involved CNS infection (Bin *et al.* 2001, Nash *et al.* 2001, Platonov *et al.* 2001). Subsequent comparisons revealed close sequence homology among strains collected from the 1996 outbreak in Romania, the 1998 outbreak in Israel, and the 1999 outbreaks in Israel, Russia and New York (Platonov *et al.* 2001, Malkinson *et al.* 2002). The New York strain has been found to be most closely related to a strain isolated during the 1998 outbreak in Israel (Lanciotti *et al.* 1999). It has been

suggested that the strains responsible for high avian mortality in Israel may have originated in central Europe during the 1996 outbreak in Romania, and have been introduced into the middle east region by migrating birds (Malkinson *et al.* 2002). Although the strain introduced into the western hemisphere is thought to have originated from the middle east or Mediterranean regions (Lanciotti *et al.* 1999), the mechanism(s) responsible are unclear.

In 1999, the first evidence of WNV observed in the western hemisphere was a cluster of eight encephalitis cases in the NYC area (CDC 1999). These were initially identified as SLEV infections based on historical arbovirus activity in the region, patient clinical history, the identification of flaviviral antibodies in serum and cerebrospinal fluid, and detection of flavivirus antigens in CNS tissue sections from suspected patients. However, PCR tests for SLEV were negative, plaque reduction neutralization testing detected only low levels of SLEV specific antibodies, and the neuropathology observed in many patients was not typical of SLEV infection (Shieh *et al.* 2000). Subsequent PCR testing and sequencing of virus isolates from dead birds and humans identified the etiological agent as WNV (Briese *et al.* 1999, CDC 1999). In August and September of 1999, a total of 62 cases of WNV infection, with 7 fatalities, were reported in New York State, with the epicenter in the borough of Queens, NYC (Mostashari *et al.* 2001, Nash *et al.* 2001).

Concurrent with the outbreak of human disease in 1999, WNV activity was detected in wild and domestic animals. In 1999, WNV neutralizing antibodies were detected in 3 percent of horses, 5 percent of dogs and 33 percent of wild birds sampled in NYC, and a total of 20 equine cases of WNV were reported statewide (Komar *et al.* 2001a, Komar *et al.* 2001b, Trock *et al.* 2001). In the Bronx Zoo, NYC, 8 percent of the captive mammals tested, and 34 percent of the captive birds tested, were seropositive for WNV neutralizing antibodies (Ludwig *et al.* 2002). In

the fall of 1999, WNV also was detected in dead birds in Connecticut and Maryland, and isolated from mosquitoes collected in New York, New Jersey and Connecticut (Anderson *et al.* 1999, Nasci *et al.* 2001).

Epizootic activity expanded in the eastern United States in 2000, with WNV was detected in mosquitoes and/or birds in 12 states, reaching as far from the 1999 epicenter as North Carolina (CDC 2000, Marfin *et al.* 2001). Detection of epidemic transmission was limited to New York, New Jersey and Connecticut, with only 21 human cases and 2 deaths reported from these states in 2000.

A rapid expansion in the geographical range and public health significance of WNV in North America was observed in succeeding years. In 2001, 66 human WNV cases with 9 deaths were reported from ten states, including Florida and Louisiana (CDC 2002a). The range of epizootic WNV transmission extended to the southern and midwestern regions of the United States, with virus detected in mosquitoes, birds and/or equines in a total of 27 states. West Nile virus was also detected in a dead bird in Ontario, Canada, and diagnosed serologically in a resident human of the Cayman Islands with no recent travel history.

In 2002, intense epidemic transmission of WNV occurred in the United States, with 4,156 confirmed human cases, and 284 deaths, reported from 39 states (O'Leary *et al.* 2004). Incidence rates were highest in the Midwest and south central states; from Mississippi and Louisiana, to Illinois and Michigan. Over 70 percent of the cases were associated with encephalitis, meningitis or meningoencephalitis, making it the largest epidemic of neuroinvasive WNV ever recorded. In Canada, a total of 414 cases, of mostly WNV neuroinvasive disease, were reported in the provinces of Ontario and Quebec (PHAC 2007). Virus was also detected in mosquitoes, birds and/or equines in a total of 44 states in the United States and 5 Canadian provinces (CDC

2002c). Evidence of WNV infection also was detected in wild birds in Jamaica and the Dominican Republic (Dupuis *et al.* 2003, Komar *et al.* 2003b). Seropositive horses were reported on the island of Guadeloupe and in several states in Mexico (Estrada-Franco *et al.* 2003, Quirin *et al.* 2004).

In 2003, 9,862 confirmed human cases of WNV were reported, with 264 deaths, in 39 states (CDC 2007a). As less than 30 percent of the WNV cases were of the neuroinvasive form, the number of neuroinvasive WNV infections was comparable with the 2002 epidemic. The high overall case count in 2003 was partly due to greatly improved surveillance for WNF cases. In Canada, a total of 1,481 human cases of WNV were reported in eight provinces and the Yukon Territories (PHAC 2007). The region of the most intense epidemic transmission of WNV shifted to the western plains and front range of the Rocky Mountains; the highest numbers of cases were observed in Texas, Colorado, Nebraska and the Dakotas in the United States, and the prairie provinces in Canada. Epizootic transmission of WNV was detected for the first time in California, Arizona, Utah and Nevada (CDC 2005a). The distribution of WNV also continued to expand in the Caribbean. In Cuba, evidence of exposure was detected in several equines and three humans (Pupo *et al.* 2006).

By 2004, WNV activity was detected in all of the contiguous states except Washington State (CDC 2007b). The intensity of transmission was much lower, with only 2,539 human cases reported for the United States. Arizona, California, Colorado, and Texas accounted for almost two-thirds of the total human WNV cases, but foci of high incidence were scattered thoughout the country (Hayes *et al.* 2005, CDC 2006). The case fatality rate was much lower in 2004, with only 100 fatalities due to WNV infection reported (CDC 2007b). There was less WNV activity in Canada as well, with only 25 human cases diagnosed (PHAC 2007). The first evidence of WNV

transmission in South America was observed in 2004, when several horses tested positive for neutralizing antibodies to WNV in Columbia (Mattar *et al.* 2005). Neutralizing antibodies to WNV also were detected in non-migratory, resident birds in Puerto Rico and Cuba (Dupuis *et al.* 2005).

The number of human infections in North America in 2005 was higher than in 2004. A total of 3,000 human cases of WNV, with 119 fatalities, were reported in the United States, and 225 cases were reported in Canada (CDC 2007c, PHAC 2007). In the United States, the highest numbers of cases were seen in California, Illinois, South Dakota, Nebraska, Texas and Louisiana. Foci of high incidence rates were observed throughout the central region of North America, from Saskatchewan and North Dakota to Louisiana and Texas. In 2005, the range of epizootic transmission continued to expand into areas of the western United States where WNV was previously undetected (CDC 2005b).

The number of WNV cases reported in the United States increased to 4,269 in 2006, with 177 fatalities, in 43 states (CDC 2007d). Similar to 2005, high incidence rates of WNV were observed in the west-central states of the U.S.A., and the prairie provinces in Canada. The region of highest epidemic transmission was Idaho, with almost a thousand cases. The range of WNV continued to expand into South America. In 2006, WNV was isolated from the brains of three horses in central Argentina (Morales *et al.* 2006). In Venezuela, serological evidence of WNV transmission in wild birds and horses also was reported (Bosch *et al.* 2007).

1.3 WNV Activity in Louisiana

Evidence of epizootic transmission of WNV in Louisiana wasn't collected until the late summer of 2001. The first detection of WNV in the state was from a dead crow collected in August from Jefferson Parish (Balsamo *et al.* 2003). West Nile virus was detected in five

additional wild birds in Jefferson Parish later that same year. In 2001, a total of ten equine cases were reported from Iberia, Calcasieu, Vermillion and Plaquemine Parishes (LDHH-OPH). A single human case was reported from Jefferson Parish in the last week of September (USGS 2002). Concurrent with the introduction of WNV, a very large outbreak of SLEV occurred in Ouachita, Richland and Morehouse Parishes, where a total of 70 human cases were reported (LDHH-OPH). In 2001, no WNV activity was detected in mosquitoes in Louisiana.

Louisiana residents experienced their largest WNV epidemic in 2002. Early WNV activity was detected in wild birds in several parishes throughout the spring, beginning in February (LDHH-OPH, Palmisano *et al.* 2005, Gleiser *et al.* 2007). Throughout the year, evidence of WNV infection was detected in several species of mosquitoes, wild birds, wild and captive mammals, horses and sentinel chickens (Ratterree *et al.* 2003, Dietrich *et al.* 2005, Godsey *et al.* 2005b, Komar *et al.* 2005, Palmisano *et al.* 2005, Gleiser *et al.* 2007). A total of 329 cases were diagnosed, with cases reported from 41 of the 64 parishes (Balsamo *et al.* 2002). Most of the cases (204) were diagnosed with neuroinvasive illness, and 24 cases were fatal. The overall incidence of neuroinvasive disease in Louisiana was 46 cases per million population, the fourth highest rate in the United States in 2002 (O'Leary *et al.* 2004). The highest incidence of neuroinvasive disease was seen in Red River, Rapides and Point Coupee Parishes, and the parishes north of Lake Pontchartrain (Balsamo *et al.* 2003).

In 2002, human infection was detected approximately one month earlier in Louisiana than reported in the northern United States (CDC 2002c), with the greatest number of human infections in the state reported during the first week of August. The timing of human cases was staggered among different foci within Louisiana, beginning with the first cases in the Parishes north of Lake Pontchartrain in June, East Baton Rouge and Ascension Parishes at the end of

June, Calcasieu, Ouachita, Orleans, Jefferson and Point Coupee Parishes in July, and Rapides Parish in August (Balsamo *et al.* 2003). In East Baton Rouge and St. Tammany Parishes, WNV was first detected in wild birds, followed by detection in mosquito pools (Palmisano *et al.* 2005, Gleiser *et al* 2007). In East Baton Rouge, the first seroconversion in sentinel chickens and first detection of WNV illness in humans occurred simultaneously. In St. Tammany Parish, the first sentinel chicken seroconversion preceded the first human WNV case by one week.

The intensity of WNV transmission in Louisiana was reduced in 2003; only 121 WNV cases were diagnosed, with 8 fatalities, in 33 parishes (LDHH-OPH). The highest incidence of human infection was in the northwestern corner of the state. A total of 64 equine cases were reported in 2003. Evidence of WNV infection also was detected in several species of mosquitoes, wild avians, wild mammals, captive alligators, and sentinel chickens (LDHH-OPH, Michaels 2003, Root *et al.* 2005, Gruzynski 2006).

In 2004, a total of 109 human cases were diagnosed in Louisiana, with 7 deaths (CDC 2007b). No foci of high human incidence of WNV were observed in the state. A total of 70 equine cases were reported.

The number of human cases of WNV increased to 171 in 2005, with 11 deaths (CDC 2007c). Similarly in 2006, the human case total was 180, with 9 deaths (CDC 2007d). In both years, high rates of human infection were observed in the parishes southeast of Baton Rouge. In 2005, the highest incidence of human infection was in Livingston Parish, with approximately 218 cases per million population. The following year, the incidence was highest in neighboring Ascension Parish, with about 252 cases per million population. In 2006, intense epidemic transmission of WNV also was observed in Tangipahoa, Washington and St. Tammany Parishes.

1.4 WNV Pathology and Epidemiology in North America

Similar to the sequence of infection in vertebrate hosts demonstrated for Dengue virus, WNV initially infects the Langerhan's dendritic cells in the skin (Byrne *et al.* 2001). Infected dendritic cells are carried to the secondary lymphoid tissues where a second round of viral replication occurs. The virus can then able to enter the circulatory system and infect the visceral organs, and possibly the central nervous system (CNS). The strain of WNV introduced into North America has been shown experimentally to be significantly more neuroinvasive in vertebrate hosts than many Old World strains (Beasley *et al.* 2002), which may explain why higher frequencies of CNS infection have been associated with WNV outbreaks in North America.

The majority of WNV infections in humans are subclinical. Of the estimated 2.6 percent of the population within the epicenter in Queens who were infected with WNV during the 1999 outbreak in NYC, only 20 percent manifested clinical disease (Mostashari *et al.* 2001). Most symptomatic individuals develop West Nile fever (WNF), a self limiting, mild febrile illness which may last several days to several months (Hayes *et al.* 2005).

In a minority of persons exposed to WNV, neuroinvasive disease may develop. In 1999, the estimated ratio of WNF to WNV neuroinvasive cases in symptomatic patients in Queens, NYC, was 30:1 (Mostashari *et al.* 2001). This ratio has varied greatly from year to year, and among different states, presumably due to differences in physician awareness and the proportion of WNF cases reported. Overall, neuroinvasive disease represents less than 1 percent of all WNV human infections in the United States. Symptoms of WNV neuroinvasive disease range from mild disorientation to coma and death (Hayes *et al.* 2005). Acute flaccid paralysis due to abnormal function of the anterior horn cells and motor axons has been observed in approximately

13 percent of patients with neuroinvasive disease (CDC 2002b, Hayes *et al.* 2005). The risk of developing of severe neuroinvasive disease and case fatality rates are higher in older age groups (Balsamo *et al.* 2003, O'Leary *et al.* 2004, Hayes *et al.* 2005). Case fatality rates for neuroinvasive WNV disease in the United States ranged from about 9 to 12 percent between 1999 and 2002 (Bernard and Kramer 2001, O'Leary *et al.* 2004). Overall case fatality rates for WNV infection have been declining since 1999, as the sensitivity of WNF case detection has increased.

Long term neurological sequelae have been observed in a significant proportion of patients with severe neuroinvasive illness. Less than 40 percent of the surviving patients hospitalized for WNV in New York in 1999 were fully recovered one year after the acute infection, and almost a third still required daily living assistance after 18 months (Klee *et al.* 2004). Long lasting sequelae was most common in patients over the age of 65. In Israel, the death rate in WNV patients two years after the acute infection was significantly elevated over the general population (Green *et al.* 2005). Several other long term sequelae associated with WNV infection have been reported, including a possible link with parkinsonism and post-polio syndrome (Sejvar 2007).

1.5 WNV Infection in Mosquitoes

A mosquito may acquire an arboviruses by horizontal transmission during blood feeding, or during copulation (venereal transmission), or by vertical transmission during egg development (transovarial transmission) or fertilization. Both venereal and vertical transmission of WNV has been demonstrated experimentally in mosquitoes (Baqar *et al.* 1993, Dohm *et al.* 2002b, Goddard *et al.* 2003, Reisen *et al.* 2006c). West Nile virus also has been isolated from males and nulliparous females collected from the field, confirming that vertical transmission of WNV does

occur under natural conditions (Miller *et al*. 2000, Farajollahi *et al.* 2005). Although the most common route of acquisition of WNV for mosquitoes under natural conditions is probably via blood feeding on viremic vertebrates, both horizontal and vertical transmission mechanisms may be important for maintenance and early season amplification of the virus (Anderson and Main 2007).

Mosquitoes serve as biological vectors of WNV by supporting both dispersal and replication of the virus. Mosquito tissues which can sustain WNV replication include the midgut and associated musculature, the CNS, the salivary glands, and the fat body (Girard *et al.* 2004). In mosquitoes which acquire the virus during blood feeding, the virus is thought to first infect the mesenteron and undergo replication, then transverse the basal lamina of the midgut, possibly through tracheal or muscle tissue conduits (Romoser *et al.* 2004). Once the virus has entered the hemocoele, the infection may disseminate to other tissues. In *Cx. quinquefasciatus*, viral replication is first seen in the muscles associated with the midgut and fat body tissue, including fat body immediately adjacent to the salivary glands (Girard *et al.* 2005). As the infection progresses, the virus may infect the CNS, particularly the first optic neuropile, and transverse the basal lamina of the salivary glands. Once replication has occurred in the salivary glands, virus may accumulate in the salivary ducts and be expelled during salivation. Once infected with WNV, a mosquito is infected for the remainder of its lifespan (Girard *et al.* 2007).

Although most arboviruses are much less damaging to arthropod cells than to vertebrate cells (Hardy 1988), some cytopathology has been observed in WNV infected mosquitoes. In *Cx. quinquefasciatus*, infection with WNV has been demonstrated to cause degeneration and apoptosis of salivary gland and midgut epithelial cells, and possibly damage to the midgut muscles and CNS (Girard *et al.* 2005, Vaidyanathan and Scott 2006, Girard *et al.* 2007).

1.6 Vector Incrimination and Vector Competence

Evidence of WNV infection has been detected in 62 of the 174 mosquito species known to occur in North America (Darsie and Ward 2005, CDC 2007e). Of these 62 species, 41 are known to occur in Louisiana, and 35 have been collected from within East Baton Rouge Parish (Chapman and Johnston 1986). Although a few of these species have been demonstrated to be important vectors of WNV, many may simply be incidental hosts of the virus, serving no significant role in the transmission or maintenance of WNV in Louisiana. The principles of vector incrimination may be used to determine whether or not a mosquito species plays a significant role in the transmission cycle of WNV, and to define the nature of the role (ie. bridge vector, enzootic vector, etc.). The four criteria required to incriminate a suspected vector are: (1) demonstration of efficient transmission of the pathogen by the suspected vectors under controlled conditions (ie. vector competence), (2) repeated demonstration that the suspected vector species, collected under natural conditions, harbors the infective stage of the pathogen, (3) demonstration of a convincing biological association, in time and space, between the suspected vector and the occurrence of clinical or subclinical infections in vertebrate hosts of the pathogen, and (4) demonstration, under natural conditions, that the suspected vector feeds upon vertebrate hosts of the pathogen, or otherwise makes effective contact with the vertebrate host (Barnett 1962, Edman and Eldridge 2000).

For successful transmission, the virus must be able to escape the blood meal, transverse the midgut to enter the hemocoele, infect the salivary glands and be expelled in the saliva during blood feeding in sufficient quantities to initiate an infection in a naïve vertebrate host. There are five broad physiological barriers which may inhibit this process; (i) the peritrophic membrane barrier, (ii) midgut barrier, (iii) the hemolymph barrier, (iv) the salivary gland barrier, and (v) the

salivary gland escape barrier (Edman 2000). The influence of these barriers on vector competence for WNV varies among different mosquito species and viral strains (Hayes *et al.* 1984, Sardelis *et al.* 2002). Infection barriers are threshold dependent on the titre of WNV ingested (Jupp and McIntosh 1970, Jupp 1974, Jupp *et al.* 1986). These infection barriers to WNV infection are also influenced by environmental temperature, mosquito age, and larval rearing conditions (Jupp 1974, Baqar *et al.* 1980, Cornel *et al.* 1993, Dohm *et al.* 2002a).

Another consideration when evaluating a potential vector is the extrinsic incubation period (EIP), ie. the period from when the time an infected blood meal is acquired to the time the mosquito becomes infective. Daily survival within many mosquito populations is quite low, so only a small fraction of females may survive beyond the second gonotrophic cycle (Milby and Reisen 1989). The EIP of WNV varies among vector species and viral strains, and is greatly influenced by environmental temperatures (Ebel *et al.* 2004, Reisen *et al.* 2006a). In southern California, Reisen *et al.* (2006a) observed that the first detection of enzootic transmission in the spring in sentinel chickens and mosquitoes occurred when increasing temperatures decrease the EIP in the primary enzootic vector, *Cx. tarsalis* Coquillett, to less than the length of two gonotrophic cycles (Reisen *et al.* 2006a). Noting that the dispersal and large outbreaks of WNV from 2002 to 2004 in the western and central United States were linked spatially and temporally with higher than average summer temperatures, Reisen *et al.* (2006a) suggested that the influence of temperature on EIP may have been a critical factor in the distribution of WNV activity in North America.

Vector competence is assessed by measuring the proportion of individuals in the arthropod population or species of interest to become infected by, and to subsequently transmit, a given strain of a pathogen (Eldridge 2000). To determine vector competence, the virus may be

introduced by feeding the mosquito a known concentration of virus in suspension, or by directly feeding the mosquito on a viremic host (Meyer *et al.* 1986a). The proportion of mosquitoes exposed that can transmit the virus is assessed after a sufficiently long EIP to allow viral dissemination and salivary gland infection. The transmission rate can be assessed *in vivo*, by feeding the mosquito on a naïve host, or *in vitro*, by inducing salivation and measuring the concentration of virus in the saliva. Additionally, the proportion of females with a disseminated infection and/or salivary gland infections can be assessed.

Several laboratory studies have examined the relative susceptibility of North American mosquito species to infection with the WNV, and their ability to successfully transmit this pathogen to a vertebrate host. The species with the highest vector competence include *Cx. erythrothorax* Dyar, *Cx. restuans* Theobald, *Cx. salinarius* Coquillett, *Cx. stigmatosoma* Dyar, *Cx. tarsalis*, *Cx. thriambus* Dyar, *Aedes albopictus* (Skuse), *Ochlerotatus atropalpus* (Coquillett) and *Oc. japonicus* (Theobald) (Sardelis *et al.* 2001, Turell *et al.* 2001, Goddard *et al.* 2002, Sardelis *et al.* 2002, Turell *et al.* 2002a, Ebel *et al.* 2005, Reisen *et al.* 2005, Reisen *et al.* 2006b). Although *Cx. pipiens* Linnaeus is generally regarded as the primary enzootic vector of WNV in the northeastern United States, it is only a moderately competent vector in the laboratory (Turell *et al.* 2000b, Turell *et al.* 2001, Goddard *et al.* 2002). Similarly, the primary enzootic vector of WNV and SLEV in many areas of the southeastern United States, *Cx. quinquefasciatus,* also has been found to be only a moderately competent vector of WNV, although there is great variability among populations (Sardelis *et al.* 2001, Goddard *et al.* 2002, Reisen *et al.* 2005). *Culex nigripalpus* Theobald, the primary vector of WNV and SLEV in parts of central and southern Florida, has been shown to exhibit a low to moderate vector competence for WNV (Sardelis *et al.* 2001), expelling only a small amount of virus during salivation (Colton and Nasci 2006).

Other North American mosquito species with a moderate, or low to moderate vector competence for WNV include *Ae. vexans* (Meigen), *Ae. aegypti* Linnaeus, *Oc. canadensis* (Theobald), *Oc. cantator* (Coquillett), *Oc. dorsalis* (Meigen), *Oc. melanimon* Dyar, *Oc. sollicitans* (Walker), *Oc. triseriatus* (Say), *Oc. trivittatus* (Coquillett), *Culiseta inornata* (Williston) and *Cs. incidens* (Thomson) (Turell *et al.* 2000b, Turell *et al.* 2001, Goddard *et al.* 2002, Tiawsirisup *et al.* 2004, Turell *et al.* 2005, Erickson *et al.* 2006, Reisen *et al.* 2006b). A few North American mosquito species have been demonstrated to be very refractory to WNV infection, including *Psorophora ferox* (von Humboldt), *Cs. melanura* (Coquillett), *Coquillettidia perturbans* (Walker), *Oc. sierrensis* (Ludlow) and *Oc. taeniorhynchus* (Wiedemann) (Turell *et al.* 2000b, Turell *et al.* 2001, Sardelis *et al.* 2001, Goddard *et al.* 2002, Turell *et al.* 2005).

1.7 Methodology for WNV Detection in Arthropod Vectors

Vector incrimination requires the detection of infective pathogen in field populations of the potential vector. Detection of arbovirus infection in vector populations also is a vital component of any surveillance program. An appropriate detection assay must be specific for the pathogen of interest, and sensitive enough to detect low levels of infection.

Regardless of the methodology used, arbovirus detection is expensive and often labor-intensive. The low infection rate of most arthropod-borne pathogens in vector populations requires that a large number of specimens must be processed to achieve a reasonable probability of detection (Gu and Novak 2004). To increase the efficiency of arbovirus testing, specimens are usually grouped into pools for testing. The true infection rate (IR) in the arthropod population can be estimated from pooled data using either the minimum infection rate (MIR), or the maximum likelihood estimation (MLE) method. Each of these estimates is biased. Depending on pool size and IR, MIR tends to underestimate IR and MLE may overestimate IR (Gu *et al.* 2004).

Calculation of MIR requires the assumption that only a single positive specimen is present in each positive pool. This will result in a very low precision estimate of IR when IR is high, and/or the pool size is large (Walter *et al.* 1980, Gu *et al.* 2003). The MLE does not require this assumption and is considered a more accurate and robust estimate of IR, provided that not all of the pools are positive for the pathogen (Chiang and Reeves 1962). To increase the precision of MLE, pool size may be reduced as infection rate increases to ensure that some of the pools tested will be negative (Gu *et al.* 2004). The methodology used to collect the arthropod samples is also an important consideration when choosing an appropriate pool size and IR estimate (Katholi and Unnasch 2006).

The standard reference method used for arbovirus detection in arthropods is virus isolation in live hosts or cell culture (Kauffman *et al.* 2003). Specific applications of the virus isolation method are also referred to as the cell culture assay, the Vero cell culture assay, and the virus plaque assay. For WNV detection, virus is allowed to replicate *in vitro* in permissive vertebrate cell lines, most often in African green monkey kidney (Vero) cells. Serial dilutions of homogenate from an individual or pooled mosquito sample are used to inoculate monolayer cell cultures. After the virus is incubated for sufficient time to allow several replication cycles, the cultures are examined for cytopathology. By counting the number of viral plaques, this assay can provide a quantitative estimate of the amount of infectious virus particles present in the sample, expressed as plaque forming units (PFU). Identification of the viral species requires a second, more specific immunological or molecular assay. This limitation has been addressed with the in situ enzyme immunoassay (EIA), which incorporates both virus isolation on cell culture and specific virus antigen detection in a single assay (Graham *et al.* 1986).

A number of assays have been developed which use viral antigen capture to detect WNV. The first was a capture enzyme EIA developed for detection of SLEV (Tsai *et al.* 1987), and later modified to detect WNV (Hunt *et al.* 2002). In this assay, two monoclonal antibodies (MAb) recognizing different epitopes of the envelope protein are used; a WNV specific MAb to capture WNV antigen (Ag) in the mosquito sample, and a flavivirus group specific MAb, conjugated with hydrogen peroxide, to detect WNV Ag bound to the capture MAb. Two commercial antigen capture assays for WNV detection based on this principle, the VecTest™ WNV antigen assay (Medical Analysis Systems, Inc., Camarillo, CA), and the Rapid Analyte Measurement Platform (RAMP®) WNV test (Response Biomedical, Corp., Burnaby, BC), are widely used in WNV surveillance programs throughout North America. An advantage of the RAMP is that it provides some quantification of viral load in the sample; the VectTest is a strictly qualitative assay.

Molecular methods used to detect the presence of WNV include traditional reverse transcription polymerase chain reaction (RT-PCR), the Taqman-based RT-PCR, nucleic acid sequence-based amplification (NASBA), and reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays (Porter *et al.* 1993, Lanciotti *et al.* 2000, Lanciotti and Kerst 2001, Parida *et al.* 2004). Currently, the most widely used method for detecting WNV RNA in mosquitoes is Taqman RT-PCR assay based on the protocol described by Lanciotti *et al.* (2000). This method uses a reverse transcriptase to amplify short sequences of the WNV genome. Complementary, fluorescent-labeled probe sequences are used to detect amplicon. Using a real time format, RT-PCR can provide relative or absolute quantification of virus in the sample (Mackay *et al.* 2002).

The virus isolation method only detects infectious virus particles. This may be advantageous when attempting to incriminate a potential vector, but a limitation for use in routine arbovirus surveillance. The rapid loss of infectivity of arboviruses during transport and processing of samples can greatly reduce sensitivity of this assay. Although WNV infectivity only declines by a factor of about 10 fold per day when held at 28ºC in cell free media (Mayo and Beckwith 2002), the infectivity of WNV and other flaviviruses in dead mosquitoes is quickly lost when held at temperatures above 4ºC (Johansen *et al.* 2002, Turell *et al.* 2002b). In contrast, WNV antigens and WNV RNA can remain stable at room temperature for weeks or months. It has been demonstrated that failure of cell culture to detect infectious Western Equine Encephalitis virus (WEEV) and SLEV in some RT-PCR positive samples is related to a reduced sensitivity when using the former detection method; virus can be detected in these samples by cell culture if they are first amplified by blind passage in insect cells (Kramer *et al.* 2002).

Another potential weakness of the viral isolation method is its lack of specificity. This can be advantageous when attempting to detect a broad range of pathogens, but it requires the addition of a second assay for specific identification. Antigen capture assays and RT-PCR are highly specific. In a blind laboratory test comparing RT-PCR, VecTest and the in situ EIA, no false positives were detected (Chiles *et al.* 2004), although only 14 negative samples were tested. Ryan *et al.* (2003) also reported that the VecTest is highly specific; no false positives were detected, even in samples containing high concentrations of SLEV, EEEV or WEEV antigens. However, the very high sensitivity of the TaqMan RT-PCR assay may increase the risk of false positives due to contamination (Shi *et al.* 2001). The development of new WNV strains may also result in a higher frequency of false negatives due to the limited ability of the TaqMan RT-PCR assay to detect even single nucleotide mutations in the sequence of the probe binding site (Papin

et al. 2004), although recently developed primer-probe sets may reduce this risk (Usuku *et al.* 2004, Tang *et al.* 2006).

The TaqMan RT-PCR assay is extremely sensitive, with a detection threshold of about 0.08 to 0.25 PFU per sample (Lanciotti *et al.* 2000, Shi *et al.* 2001, Kauffman *et al.* 2003, Sawabe *et al.* 2006). In a comparison of techniques for detecting WNV in field collected mosquito pools, it was found that TaqMan RT-PCR was significantly more sensitive than virus isolation or VecTest; virus isolation only detected WNV in 74 percent of Taqman positive pools, and only 60 percent of Taqman positive pools were positive by VecTest (Nasci *et al.* 2002). A similar study found that VecTest only detected WNV in about 50 percent of field collected mosquito pools that were positive by the TaqMan assay (Lampman *et al.* 2006). For antigen capture assays, it was reported that the RAMP test is 100 fold more sensitive than the VecTest for detecting WNV in mosquitoes (Burkhalter *et al.* 2006). Sensitivity of both the virus isolation and RT-PCR assays are negatively associated with mosquito pool size (Yuill 1969, Lanciotti *et al.* 2000, Johansen *et al.* 2002).

In practice, all three approaches to WNV detection in mosquitoes have positive and negative attributes. The viral isolation method requires a high level of training and access to BSL3 facilities, is expensive, is not suitable for processing large numbers of samples, and takes several days to obtain a result. Molecular methods provide a result much more quickly than cell culture and are more compatible with automated, high-throughput screening of samples, but require some experience and training. Commercial antigen capture assays, particularly the VecTest, require very little equipment or training, and provide a result much more quickly than any other WNV detection method (Burkhalter *et al.* 2006).

1.8 Mosquitoes Vectors of WNV in North America and Louisiana

The species implicated as important vectors of WNV are primarily within the genus *Culex* (Hayes 1989, Hubalek and Halouzka 1999). Based on vector competence data, frequency of WNV infection, bloodfeeding habits, and other criteria, the species implicated as the most important enzootic vectors and/or bridge vectors of WNV in North America include *Cx. pipiens, Cx. quinquefasciatus, Cx. restuans, Cx. tarsalis, Cx. stigmatosoma, Cx. erythrothorax, Cx. salinarius* and *Cx. nigripalpus* (Turell *et al.* 2005). In the northeastern United States and eastern Canada, *Cx. pipiens* and *Cx. restuans* have been implicated as the primary enzootic vectors of WNV (Andreadis *et al*. 2001, Bernard *et al.* 2001, Nasci *et al.* 2001, Condotta *et al.* 2004, Ebel *et al.* 2005). In the prairie provinces of Canada, and much of the western United States, the primary enzootic WNV vector is *Cx. tarsalis* (Naugle *et al.* 2004, Reisen *et al.* 2004b, Bell *et al.* 2005). In the southwestern states, *Cx. quinquefasciatus* is an important enzootic vector in urban areas (Reisen *et al.* 2004b, DiMenna *et al.* 2006). *Culex quinquefasciatus* is also the primary enzootic vector of WNV in the southeastern United States (Godsey *et al.* 2005a, Godsey *et al.* 2005b). In southern and central Florida, *Cx. nigripalpus* is considered the most important enzootic vector of WNV (Rutledge *et al.* 2003). Other species suspected of acting as important bridge vectors of WNV in North America include *Aedes albopictus, Ochlerotatus triseriatus* and *Oc. japonicus* (Turell *et al.* 2005).

Culex quinquefasciatus has been implicated as the primary vector of WNV in Louisiana (Palmisano *et al.* 2005, Gleiser *et al.* 2007). It is also the primary vector of SLEV in the southeastern United States (Chamberlain *et al.* 1959). This mosquito is very common in both urban and rural habitats in East Baton Rouge Parish, is active year round, and can reach very high densities during the warmer months of the year (Chapman and Johnson 1985). The majority

of WNV infections detected in mosquitoes collected from urban habitats in Louisiana are in pools containing female *Cx. quinquefasciatus* (Arbonet 2004, Godsey *et al.* 2005b, Palmisano *et al.* 2005, Gleiser *et al.* 2007).

Two other common *Culex* species in Louisiana have been implicated as important enzootic vectors of WNV in other regions of the United States. *Culex nigripalpus* is the primary enzootic and epidemic vector of SLE and WNV in Florida (Chamberlain *et al.* 1964, Rutledge *et al.* 2003). This species is most abundant during the late summer and autumn months (Chapman and Johnson 1985), and may contribute to late season enzootic and epidemic transmission of WNV in Louisiana. The other species, *Cx. restuans*, is considered an important enzootic vector of WNV in the northeastern United States (Ebel *et al.* 2005). This species is most abundant during the winter and early spring (Chapman and Johnson 1985), and may play a role in the overwintering and early season amplification of WNV in Louisiana.

Several mosquito species common to Louisiana have been implicated as bridge vectors of WNV. *Culex salinarius* is considered a potentially important bridge vector of Eastern Equine Encephalitis virus (EEEV) and WNV (Vaidyanathan *et al.* 1997, Turell *et al.* 2005). In Louisiana this species can be active year round (Chapman and Johnson 1985). West Nile virus has been detected in pools containing *Cx. salinarius* collected from several parishes Louisiana (Arbonet 2004, Godsey *et al.* 2005b, Palmisano *et al.* 2005). *Aedes albopictus* and *Oc. triseriatus* have also been implicated as epidemic vectors of WNV (Turell *et al.* 2005, Erickson *et al.* 2006). These species are two of the most common container breeding species in Louisiana, are abundant in suburban habitats, and are diurnal bloodfeeders (Chapman and Johnson 1986, Schreiber *et al.* 1988, Barker *et al.* 2003).

1.9 Vertebrate Hosts of WNV in North America and Louisiana

Vertebrate hosts are essential to the amplification and maintenance of most arboviruses. They may also play a critical role in the dispersal of WNV (Rappole *et al.* 2000, Malkinson *et al.* 2002, Petterson *et al.* 2003). Athough a very broad range of vertebrate species are susceptible to infection with WNV (van der Meulen *et al.* 2005), only a minority sustain a high enough viremia, for a sufficient duration, to serve as a significant source of infection for a mosquito vector population. In addition to reservoir competence, the contribution of a specific host to WNV maintenance, dissemination and/or amplification at a given location depends upon the spatial and temporal distribution of the hosts, relative to the feeding behavior of the local vector species. Avian hosts that are reproductively active during the period of epizootic transmission of WNV may be especially important. Nestling birds represent an immunologically naïve host for WNV, and a passive host for mosquito vectors (Blackmore and Dow 1958).

Wild birds are considered the principle hosts of WNV (Hubálek and Halouzka 1999, Malkinson and Banet 2002). Since 1999, evidence of WNV infection has been detected in over 300 species of wild and captive birds in North America (CDC 2007f). Experimental infection studies with WNV have demonstrated that the most competent avian hosts are in the order Passeriformes (Komar *et al.* 2003a, Langevin *et al.* 2005). Several chadriiform and raptor species also have been shown to develop high WNV titres. Most Anseriformes and Columbiformes species have been found to be only weakly competent reservoir hosts for WNV (Swayne *et al.* 2001, Komar *et al.* 2003a, Allison *et al.* 2004, Reisen *et al.* 2004a). The least competent avian hosts are in the orders Psittaciformes and Galliformes (Komar *et al.* 2003a, Langevin *et al.* 2001).

One of the characteristics of the WNV strain introduced into North America is a high pathogenicity in certain avian hosts, particularly corvids (Anderson *et al.* 1999). Members of the avian family Corvidae have been demonstrated to be particularly susceptible to infection with WNV (Komar *et al.* 2003a), and exposure rates in live and dead corvids are often used to detect early season WNV activity (Eidson *et al.* 2001, Lindsay *et al.* 2003). In urban areas in southern California, WNV infection rates in humans and mosquitoes are significantly correlated with American Crow density and WNV infection (Reisen *et al.* 2006d). The American Crow may be very important in the dispersal of WNV in urban environments (Ward *et al.* 2006). Significant declines in American Crow populations have been attributed to WNV mortality is several areas of the United States (Caffrey *et al.* 2003, LaDeau *et al*. 2007). However the primary WNV enzootic vectors in the eastern United States, *Cx. quinquefasciatus* and *Cx. pipiens*, rarely feed on American Crows (Apperson *et al.* 2002, Apperson *et al.* 2004, Molaei *et al.* 2006, Molaei *et al.* 2007). The high rate of exposure in large corvids, such as the American Crow and the Fish Crow, may be primarily from consumption of WNV infected material. It has been demonstrated that corvids can acquire the virus orally by preying or scavenging on infected birds (Komar *et al.* 2003a).

Other corvid species may be important sources of infection for mosquito vectors. The Blue Jay has been demonstrated in the laboratory to be an extremely competent reservoir host for WNV, developing a peak viremia of up to $10^{12.1}$ PFU / ml of serum (Komar *et al.* 2003a). Similarly maximum viremia levels in the Western Scrub Jay may reach greater than 10^{9} PFU / ml of serum (Reisen *et al.* 2005). These virus concentrations may be sufficient to infect even weakly competent mosquito vectors. The Blue Jay has been implicated as a possible winter maintenance host in the southern United States (Tesh *et al.* 2004). High WNV seroprevalence

rates have been reported in Blue Jays from St. Tammany and East Baton Rouge Parishes, Louisiana (Komar *et al.* 2005, Gruszynski 2006, Gleiser *et al.* 2007).

In New York, New Jersey, Connecticut and Maryland, the American Robin is one of the most frequent hosts of *Cx. pipiens*, the primary enzootic vector in the northeastern United States (Apperson *et al.* 2002, Apperson *et al.* 2004, Kilpatrick *et al.* 2006a, Molaei *et al.* 2006). In Maryland, North Dakota, Minnesota, Illinois moderate to high WNV seroprevalence rates have been reported in this species (Bell *et al.* 2006, Beveroth *et al.* 2006, Kilpatrick *et al* 2006b). The American Robin has been demonstrated to be a moderately competent reservoir for WNV (Komar *et al.* 2003a), and may be one of the principal amplifying hosts in urban areas in the northeastern United States.

High WNV seroprevalence rates have been measured in Northern Cardinal populations throughout the eastern and central United States (Komar *et al.* 2001b, Godsey *et al.* 2005a, Beveroth *et al.* 2006, Gibbs *et al.* 2006, Molaei *et al.* 2007). In Louisiana, the Northern Cardinal had been shown to have one of the highest rates of exposure to WNV of all wild bird species sampled, with reported seroprevalence rates of up to 48 percent during the summer months (Komar *et al.* 2005, Gruszynski 2006, Gleiser *et al.* 2007). This species is considered a moderately competent reservoir host for WNV. For experimentally exposed birds, Komar *et al.* (2005) reported mean peak viremias of $10^{5.6}$ PFU / ml of serum in cardinals that survived the infection, and mean peak viremias of greater than $10^{9.4}$ PFU / ml of serum in cardinals with fatal infections (Komar *et al.* 2005). Northern Cardinals are very abundant in edge habitats associated with urban and suburban areas throughout the eastern and central United States (Burhans and Thompson 2006, Leston and Rodewald 2006).

A very common peridomestic species, the House Sparrow has been implicated as an important amplifying host of WNV in urban areas throughout the United States (Komar *et al.* 2001b, Godsey *et al.* 2005, Nasci *et al.* 2001, Reisen *et al.* 2006d, Molaei *et al.* 2007). Significant levels of WNV neutralizing antibodies have been detected in House Sparrows collected from Louisiana (Komar *et al.* 2005, Gruszynski 2006, Gleiser *et al.* 2007). The House Sparrow is considered a moderately to highly competent reservoir host of WNV (Komar *et al.* 2003a, Komar *et al.* 2005). Experimentally infected birds can produce peak viremias greater than 10^{10} PFU / ml of serum (Komar *et al.* 2003a, Langevin *et al.* 2005).

Several other passeriform birds common to Louisiana, including the Red-winged Blackbird, the Common Grackle and the Northern Mockingbird, are frequently exposed to WNV, and have demonstrated a moderate or high reservoir competence (Komar *et al.* 2003a, Komar *et al*. 2005, Gruszynski 2006, Gleiser *et al.* 2007). Other species that have not been evaluated as reservoir hosts, but have been reported to have high WNV seroprevalence rates in Louisiana include the Brown Thrasher, the Carolina Wren, the Hermit Thrush and Red-bellied Woodpecker (Komar *et al.* 2005, Gruszynski 2006). Mourning Doves, though exhibiting fairly high levels of neutralizing antibodies to WNV in wild bird serosurveys conducted in Louisiana (Komar *et al*. 2005, Gruszynski 2006), are relatively incompetent reservoir hosts (Komar *et al.* 2003a).

Most mammal species evaluated as amplifying hosts, including domestic horses, cats, dogs and pigs, have been demonstrated to be refractory or very weakly competent for WNV (Bunning *et al.* 2002, Austgen *et al.* 2004, Teehee *et al.* 2005). Reservoir competence for WNV has been demonstrated in a small number of rodent and lagomorph species. Eastern Cottontail Rabbits infected after exposure to infected mosquitoes can develop a sufficient level of

circulating virus to infect *Cx. salinarius* and *Cx. pipiens* (Tiawsirisup *et al.* 2005). California Fox Squirrels, Eastern Chipmunks, and Golden Hamsters can develop a WNV viremia of 5 $\log_{10}$PFU / ml or higher (Xiao *et al.* 2001, Padgett *et al.* 2007, Platt *et al.* 2007), which is sufficient to infect competent mosquito vector species. However, most mammalian species are unlikely to serve as important amplifying hosts of WNV. It has been suggested that abundant mammalian hosts may be zooprophylactic by diverting vectors from feeding on competent vertebrate hosts (Hess and Hayes 1970).

Although WNV infection has been documented in the several wild and captive reptile species (Nir *et al.* 1969, Miller *et al.* 2003, Steinman *et al.* 2006, Jacobsen *et al.* 2005, Gruszynski 2006), few studies have implicated poikilotherms as important to the epizootiology of WNV. Common Garter Snakes innoculated with a high WNV dose (10^5 PFU) only developed low levels of circulating virus (Steinman *et al.* 2006). When inoculated with 2 x 10^3 PFU of WNV, Green Iguanas and North American Bullfrogs developed viremias ($<10^{3.5}$ PFU / ml of serum) insufficiently low to infect a mosquito vector, and no viremia was detected in Red-eared Sliders and Florida Garter Snakes (Klenk and Komar 2003). However, high levels of circulating virus have been detected in the American Alligator after experimental inoculation with WNV (Klenk *et al.* 2003).

1.10 Mosquito Host Seeking Behavior, Blood Feeding and Oviposition

Adult females of most mosquito species are anautogenous; obtaining the protein required for yolk synthesis and egg development by hematophagy. Successful location of a blood meal source requires significant expenditure of energy reserves, exposes the mosquito to unfavorable environmental conditions, and can take the mosquito a long distance from suitable oviposition sources. Blood feeding involves a high risk of mortality from host defensive behaviors, and

reduced mobility may leave engorged females vulnerable to predation. Therefore blood feeding in mosquitoes requires highly specialized behavioral and physiological mechanisms to efficiently locate a suitable host, to avoid triggering host defenses, and to rapidly imbibe and digest large volumes of blood.

Host seeking behavior in biting flies may be divided into three components: (i) appetitive search, (ii) activation and orientation, and (iii) attraction (Sutcliffe 1987, Dodd and Burgess 1995). Appetitive search is defined as the non-orientated behavior which functions to bring the host seeking female into contact with host stimuli. Once host stimuli are received, oriented host location behavior is activated. Activation / orientation cues are primarily olfactory, but may include visual cues. Once the female is brought in close contact with a potential host, shorter range olfactory and visual cues determine whether contact with the host will occur.

In all mosquito species host seeking behavior is expressed according to a circadian rhythm. In most important culicinae vectors with a crepuscular or nocturnal pattern of host seeking, including *Cx. salinarius*, *Cx. nigripalpus* and *Cx. tarsalis*, peak flight activity typically occurs within one to three hours after sunset, and sometimes a second smaller peak in activity is observed shortly before sunrise (Provost 1969, Carroll and Bourg 1977, Slaff and Crans 1981, Meyer *et al.* 1986b, Reisen *et al.* 1997, Reddy *et al.* 2007). In *Cx. pipiens*, circadian fluctuations in host seeking activity don't appear to be associated with a change in olfactory sensitivity (Bowen 1992). A number of other intrinsic factors influence the expression of host seeking behavior in mosquitoes, including chronological and physiological (reproductive) age, nutritional status, mating status and diapause (Jones and Gubbins 1979, Klowden 1986, Klowden *et al.* 1988).

Carbon dioxide is considered a general host kairomone for many hematophagous arthropods, and is frequently used to increase the number of host seeking females in mosquito traps. Gilles (1980) proposed that CO_2 has at least 2 modes of action: 1) as an attractant, and 2) to augment the activity of other host stimuli. Carbon dioxide has been shown to function as a general flight activator by increasing the frequency and duration of mosquito flight (Daykin *et al.* 1965). Orientation to the source is facilitated by stimulating an increase in the frequency and angle of turns during flight when the female leaves the CO_2 plume, but not when entering one. This orientation (optomotor anemotaxis) is only observed in the presence of a pulsed gradient of CO_2 in a moving airflow (Gilles 1980, Cooperband and Cardé 2006). Carbon dioxide also sensitizes host seeking females by significantly increasing their detection threshold to other host stimuli (Dekker *et al.* 2005).

A number of volatiles associated with vertebrate hosts have been examined as potential host cues for mosquitoes. Octenol enhances the trap catches of several species when used with a CO_2 bait, though not for most *Culex* species, and few mosquito species exhibit a response to octenol bait alone (Takken and Kline 1989, Kline *et al.* 1990, Kline and Mann 1998, Mboera *et al.* 2000, Rueda *et al.* 2001). L-lactic acid, a major component of human sweat, has been shown to be attractive to several anthropophilic species including *Aedes aegypti* and *Anopheles gambiae* (Acree *et al.* 1968, Dekker *et al.* 2002), but repellent to other species, such as *Ae. albopictus* and *Culex (Melanoconion)* spp. (Kline *et al.* 1990, Shirai *et al.* 2001). Ammonia has been shown to be attractive to *An. gambiae* (Smallegange *et al.* 2005), and to *Ae. aegypti* when in combination with lactic acid (Geier *et al.* 1999). Mboera *et al*. (2000) failed to detect any attraction of *Cx. quinquefasciatus* to acetone or butyric-acid. Butanone has been found to be attractive to a few mosquito species, but repellent to most (Kline *et al.* 1990, Kline and Mann 1998).

The sensitivity of specific mosquito taxa to certain host kairomones has been associated with host specificity. Russell and Hunter (2005) reported that the total number of specimens of two ornithophilic mosquito species (*Cx. pipiens* and *Cx. restuans*) collected by CO_2 baited light traps was significantly increased by the addition of crow uropygial gland secretions as an attractant. These bird odors had no significant effect on the number of *Ae. vexans* (a mammal-feeding species) collected. Uropygial gland extracts from other avian species have been shown to be specifically attractive to certain ornithophilic black fly species (Fallis and Smith 1965). Allen *et al.* (2006) reported that a significantly greater number of *Ae. aegypti, Cx. nigripalpus* and *Cx. quinquefasciatus* were attracted to chicken feather extracts than to controls, but this attraction was not observed with *Cx. tarsalis*. Human foot odor has been shown to be attractive to anthropophilic populations of *Culex quinquefasciatus* in Africa (Mboera *et al.* 2000), and a number of mammal-feeding mosquito species in Florida (Kline 1998). It has been suggested that interspecific differences in sensitivity to CO_2 may be related to host preference. Mosquito species that prefer large hosts or feed opportunistically typically respond to higher concentrations of CO_2 than mosquito species that preferentially feed on small hosts (Reeves 1990, Mboera and Takken 1997).

Intra-specific differences in the response to certain olfactory host cues also have been correlated with regional variability in host preference between mosquito populations. In Australia, *Cx. annulirostris* populations from two different regions differed significantly in their response to chicken and guinea pig odors in the lab, and their feeding rate on these two hosts in the field (Williams *et al.* 2003).

Visual cues recognized by host seeking mosquitoes include color, shape, size and movement (Browne and Bennett 1981). Visual cues are of particular importance for diurnally

active species, although it was demonstrated that *Anopheles minimus* females can respond to illumination levels as low as 1.5 to 2.4 x 10^{-4} candela per square meter, or 25% that of starlight (Muirhead-Thomson 1940). For most mosquito species, dark colors with a low reflectance tend to be more attractive than light colors (Browne and Bennett 1981). Black and brown targets have been shown to be very attractive to *Cx. quinquefasciatus*, under either natural or ultraviolet light (Wen *et al.* 1997). Silhouettes of larger objects, such as woodland edges, are thought to be used by nocturnal and crepuscular mosquitoes for long-range orientation during appetitive flight (Bidlingmayer and Hem 1981, Bidlingmayer 1994). During this phase, large, dark and non-reflective objects are particularly attractive to host seeking females (Bidlingmayer and Hem 1979). In many species, visual cues are important during short range host seeking behavior; both for evaluation of host suitability and for selection of feeding sites on the host.

Both host body heat and moisture are detected by mosquitoes at very short distances. In the laboratory, *An. gambiae* exhibits a stronger response to relative humidity that is rising, than falling or static relative humidity (Takken *et al.* 1997). Gillett and Connor (1976) demonstrated that host seeking females can discriminate host temperature; when *Ae. aegypti* were exposed simultaneously to artificial blood sources at two different temperatures (38 and 41° C), significantly greater numbers fed on the meal at the higher temperature.

Once a suitable host is found, the female mosquito will land and find a suitable feeding site. Although specific landing site preferences have been observed on larger hosts (Knols *et al.* 1994, De Jong and Knols 1995), landing site selection on small hosts may be more indiscriminant (Walker and Edman 1985). Once a suitable feeding site has been located, the female begins probing in response to host cues such as heat, surface texture, olfactory stimuli and moisture levels (Friend and Smith 1977). As the fascicle is inserted into the host skin, blood

vessels are lacerated resulting in hemorrhaging. Probing continues for approximately 1-2 minutes, or until a suitable blood supply is located, usually by canulating a venule or arteriole with the fascicle (Clements 1992). In *Culex* and *Aedes* species, gorging is stimulated by ADP and ATP released by platelets (Galun *et al* 1993). Salivation begins during probing and continues throughout the entire blood feeding process (Golenda *et al.* 1995).

Significant differences have been observed in the blood feeding behavior of different mosquito groups. Ribeiro (2000) reported that *Cx. quinquefasciatus* spent a much greater time probing and feeding on a human or mouse than *Ae. aegypti* or *An. albimanus*, but no significant differences in probing behavior were observed when the mosquitoes were fed on a chicken. The author suggested that members of the genus *Culex* are less adapted to feeding on mammalian hosts than *Aedes* or *Anopheles* species.

Once a female mosquito has fully engorged, host seeking behavior is suppressed. In *Ae. aegypti*, suppression of host seeking behavior is mediated first by a signal from stretch receptors in the midgut (Klowden and Lea 1978), followed 12 to 24 hours post-feeding by the release of the neuropeptide, Aea-HP-I (Klowden and Lea 1979, Brown *et al.* 1994). Depending on the species, fully engorged females may imbibe one to three times their body mass in blood (Woodward and Chapman 1965). Although flight activity may be impaired immediately after engorgement, a significant proportion of the blood meal mass is excreted within 2 hours after feeding (Boorman 1960), allowing recently blood fed females to disperse to suitable resting sites during digestion and egg development (Edman and Bidlingmayer 1969). Digestion of the blood meal may require 2 to 4 days, depending on the species, reproductive status and environmental conditions (Downe *et al.* 1963, Edman 1970, Reiter *et al.* 1990). Suppression of host seeking behavior is maintained until successful oviposition (Klowden and Lea 1979).

After maturation of the oöcytes, gravid females become responsive to oviposition-site stimuli (Klowden and Blackmer 1987). These include many visual, physical, and olfactory cues associated with potential oviposition sites. Conspecific oviposition pheromones have also been described in a number of mosquito species, including *Cx. quinquefasciatus*, *Cx. tarsalis* and *Cx. salinarius* (Osgood 1970, Andreadis 1977, Bruno and Laurence 1979). Similar to the expression of host seeking behavior, oviposition behavior in most species follows a circadian cycle. In most *Culex* species, peak ovipositional activity occurs during the early scotophase (Suleman and Shirin 1981), or ovipositional activity is bimodal, with ovipositional peaks in the early and late scotophase (Logan and Harwood 1965). After oviposition, females will resume host seeking behavior (Klowden and Lea 1979).

1.11 Host Defensive Behavior, Blood Feeding Success and Multiple Blood Feeding

The defensive behavior of vertebrate hosts to mosquito feeding pressure can greatly reduce blood feeding success; both the proportion of females that are able to obtain blood and the volume of blood obtained by females that successfully feed (Edman and Kale 1971, Hodgson *et al.* 2001). The intensity of these defensive behaviors is dependent on the host species, as well as the size, age, and health of the host (Day and Edman 1984a, Edman and Scott 1987). Generally, small host species tend to be more defensive than larger host species, with many of the smaller passerine birds and rodents exhibiting the most intense defensive behaviors, such as body and head shaking (Edman *et al.* 1974). The concordance of the mosquito and host circadian activity cycles has been negatively correlated with blood feeding success. Blood feeding success is greatest on host species that are normally inactive during the period when the mosquito is actively host seeking (Day and Edman 1984b). For many host species, mosquito density has been positively associated with host defensive behavior intensity and negatively associated with

mosquito feeding success (Edman *et al.* 1972, Waage and Nondo 1982, Walker and Edman 1986, Anderson and Brust 1996).

Except for some of the Anophelinae, most anautogenous mosquito species are considered to exhibit gonotrophic concordance; ie. only one blood meal is acquired during each gonotrophic cycle. However multiple blood feeding events may occur in a single gonotrophic cycle as a result of interrupted feeding. Females prevented from fully engorging due to host defensive behavior may attempt to re-feed on the same host, or may be diverted to a different host (Hodgson *et al.* 2001). Multiple blood meals may be cryptic (obtained from multiple hosts of the same species) or patent (obtained from multiple hosts of different species) (Boreham and Garrett-Jones 1973).

The probability of an engorged female attempting to re-feed is dependent on the size of the first blood meal, and the duration between host contacts. Klowden and Lea (1978) reported that *Aedes aegypti* females which imbibed less than 2.5µl of blood continued to respond to host cues and attempted to re-feed when offered a host within 1 hr of obtaining the first meal. The majority of *Cx. nigripalpus* imbibing a half-full or smaller blood meal attempted to re-feed when offered a second meal with 6 hours of the first meal (Edman *et al.* 1975). Fewer females attempted to re-feed when the second blood meal was offered 12 to 24 hours after the first meal.

Anderson and Brust (1995) detected multiple blood meals in an average of five percent of engorged *Cx. tarsalis, Cx. restuans* and *Cx. nigripalpus* collected in box traps containing two quail marked with rubidium or cesium. A number of field studies have suggested that multiple blood feeding occurs much more frequently than predicted from controlled experiments. Cupp and Stokes (1976) detected multiple feedings in 13 percent of engorged *Cx. salinarius* collected from Jefferson Parish, Louisiana. Multiple blood meals were detected in over 36 percent of engorged *Cx. salinarius,* and almost 22 percent of engorged *Cx. tarsalis*, collected from several

sites in Kansas (Edman and Downe 1964). Almost 40 percent of engorged *Cx. tritaeniorhynchus* collected from rural villages in southern India were shown to have fed on more than one host (Arunachalam *et al.* 2005). Multiple blood feeding was detected in over 40 percent of engorged *Cx. tarsalis* collected in Washington state (Bang and Reeves 1942). However, in all these studies, multiple patent blood meals were detected using immunological assays which have been demonstrated to have poor specificity (Washino and Tempelis 1983). High multiple feeding rates observed in these studies may be in part due to cross reactivity among samples with multiple host antisera.

1.12 Mosquito Host Preference and Methods Used to Assess Host Range

Host preference is the relative attractiveness of a host species to a mosquito, and is controlled by the intrinsic response of the mosquito to specific host cues. Host range may be defined as the observed pattern of host utilization of a mosquito population. The host range of a mosquito species is primarily a function of: (i) intrinsic host preference of the mosquito, (ii) defensive behavior of available host species, and (iii) abundance of available host species. Available hosts are defined as those vertebrate species present in the same habitat at the same time as the vector. Although there is evidence of spatial variation in host preference between mosquito populations (Williams *et al.* 2003), and of temporal changes in defensive behavior associated with host age (Kale *et al.* 1972), the greatest influence on intra-specific variability in the host range of most mosquito species is probably due to local and regional differences in host vertebrate abundance and diversity.

Tempelis (1989) described 9 basic host utilization patterns for mosquitoes. The first five, characterized as restricted feeding patterns, includes mosquitoes that feed almost exclusively on: (i) large mammals, (ii) birds, (iii) reptiles, (iv) amphibians, or (v) fish. An additional three

feeding patterns were characterized as opportunistic, and include mosquitoes that: (i) feed readily on mammals and birds, (ii) feed readily on mammals, birds and poikiotherms, or (iii) feed primarily on birds in spring and early summer, shift to feeding more frequently on mammals in late summer, and then shift back to feeding primarily on birds in the fall. The ninth feeding pattern was described as mosquito species which exhibit a restricted feeding pattern in one geographical area, and feed opportunistically in another area.

Forage ratio (FR) is a measure of utilization of a resource by an organism, relative to the abundance of that resource in the environment. It has been used to describe resource utilization patterns for a variety of predators, herbivores and parasites (Owen-Smith and Cooper 1987, Kam *et al.* 1997, Deudero and Morales-Nin 2001, Agrela *et al.* 2002, Rikardsen and Sandring 2006). In mosquito host utilization studies, FR may be used to separate the influence of host abundance from the attractiveness and/or acceptability of the host. The FR calculates the proportion of engorged mosquitoes that fed on a specific host species divided by the proportion that the host species represents of the total number of available hosts in the study area (Hess *et al.* 1968). A FR significantly greater than one is considered to indicate that the mosquito is exhibiting selective preference for that host species. A FR significantly less than one is considered to indicate that the mosquito is exhibiting selective avoidance of the host. Disadvantages of this host preference model include the requirement of quantitative host abundance data and the neglect of factors such as host availability and accessibility (Edman 1971).

A more complex model, the feeding index (FI), was developed to overcome the some of the restrictions and shortcomings of the FR (Kay *et al.* 1979). This model uses relative (rather than quantitative) host abundance data, and incorporates factors known to influence host feeding patterns, such as host size, host defensive behavior, and spatial and temporal concurrence of the

host and the vector. The FI is defined as the proportion of feedings on one host species, relative to another host species, divided by the expected proportion of meals from both host species based on the fore mentioned factors. Although the FI continues to be used to describe host utilization in mosquitoes (Hassan *et al.* 2003, Cupp *et al.* 2004a), it has been suggested that the FI method may be of little value (Service 1993). Due to the complexity of factors influencing host selection, enormous variability in the FI estimate is observed when a single factor is added or removed from the model (Kay *et al.* 1979).

A third measurement of host utilization is the human blood index (HBI), which is an estimate of the proportion of blood meals taken from humans. This estimate has very specific applications; typically to describe the transmission of pathogens by highly anthropophilic vectors where humans are the primary vertebrate reservoir, such as with the vectors of human malaria (Lardeux *et al.* 2007).

1.13 Host Blood Meal Identification Methods

A variety of methods are available for determining the host specificity of hematophagous arthropods. The earliest studies relied primarily on behavioral observations of vectors and the use of baited traps to determine host preference. However, biases associated with these methods have limited their use to specific applications (Washino and Tempelis 1983). Components of blood present in the midgut of hematophagous arthropods can be used to identify the source of the meal by immunology or molecular based methods (Washino and Tempelis 1983, Sato *et al.* 1992, Ngo and Kramer 2003). Serum albumin and complement protein (C3) have been identified in the mosquito midgut over 24 hours after ingestion, and IgG and IgM can be detected for several days (Tesh *et al.* 1988). Host mitochondrial DNA (mtDNA) has been detected in blood fed

mosquitoes for up to 1.5 to 7 days post-feeding (Lee *et al.* 2002, Ngo and Kramer 2003, Oshaghi *et al*. 2005).

A number of immunology based methods have been used to detect host specific proteins in blood fed mosquitoes. The earliest methods utilize a precipitin reaction (Bull and King 1923). The most common precipitin test used is the modified capillary tube precipitin technique (CPT) developed by Tempelis and Lofy (1963). In this method, equal amounts of antisera, containing polyclonal antibodies against general host groups, and supernant from the homogenized abdomen of a blood fed mosquito, are added to a glass capillary tube. A positive reaction is indicated by a cloudy precipitation at the antisera:supernant interface. This method was used to broadly screen blood meals between hosts at the order and family level (Tempelis and Galindo 1975, Savage *et al.* 1993, Niebylski *et al.* 1994). However, the poor sensitivity and specificity of precipitin tests limits their ability to distinguish between blood meals from specific hosts (Washino and Tempelis 1983).

Another common precipitin test is the gel diffusion technique (GDT) (Crans 1969). In this assay, antisera, sample homogenates and control antigens are placed into wells cut into an agarose gel coating a glass slide (Bheema Rao 1984, Collins *et al.* 1986). The antisera and antigens diffuse out into the gel and positive reactions are identified by the formation of a precipitin band between a blood meal sample and homologous antisera. Although the GDT provides a permanent record, it may be even less sensitive than the CPT (Tempelis 1975). However, both the GDT and CPT are still used for host blood meal identification due to their low cost and ease of use, particularly when access to modern laboratory facilities may be limited (Gomes *et al.* 2003, Van Den Hurk *et al.* 2003, Samuel *et al.* 2004).

Enzyme-linked immunosorbent assays (ELISA) have also been used to identify host blood meals. These assays include direct (Edrissian *et al.* 1985), indirect (Burkot *et al.* 1981) and sandwich (Service *et al.* 1986) ELISA methods. Most blood meal ELISA protocols have used polyclonal antibodies to detect serum antigens in the blood meal. In the 'sandwich-B' Enzyme-Linked Immunosorbent Assay (ELISA), commercially available horseradish peroxidase labeled (HRP) heavy chain, and heavy and light chain fragments, specific against IgG of a variety of mammalian and avian hosts, are used as detection antibodies (Chow *et al.* 1993). Although specificity and sensitivity is greatly improved over previous blood meal ELISA protocols, only a small number of specific hosts can be detected using the limited number of commercially produced, HRP labeled antibodies available.

Species identification by means of the differences in cytochrome B (*cytb*) gene sequence has been applied to a wide range of phylogenetic and forensic studies (Zehner *et al.* 1998, Halanych *et al.* 1999, Parson *et al.* 2000, Slowinski and Keogh 2000, Pfeiffer *et al.* 2004). There are advantages to using mtDNA markers, rather than nuclear DNA markers, for species identification. Multiple copies of mtDNA are present in eukaryotic cells, increasing the sensitivity of the assay and allowing amplification even from very small samples of degraded material (Branicki *et al.* 2003). The rate of evolutionary substitution in mtDNA is higher than nuclear DNA (Brown *et al.* 1979), which increases the specificity of the assay. A single set of primers directed at conserved regions can be used to amplify homologous segments of mtDNA from a wide range of vertebrate hosts (Kocher *et al.* 1989), without amplification of the corresponding invertebrate vector mtDNA (Boakye *et al.* 1999). For many vertebrate species, a partial or entire *cytb* gene sequence has been described and is available in existing DNA sequence databases. Several approaches have been developed to distinguish host species based

on differences in the *cytb* gene sequence of host mtDNA, including: (i) species or group specific primers, (ii) direct sequencing, (iii) the PCR-heteroduplex assay, and (iv) the terminal restriction fragment length polymorphism assay.

The first approach uses species or group specific primers to amplify a portion of the *cytb* gene by polymerase chain reaction (PCR). This method has been used to distinguish between mammalian and avian blood meals, and among blood meals from different avian orders (Ngo and Kramer 2003). This approach has also been used to distinguish blood meals from multiple mammalian hosts using species-specific primers for the *cytb* gene in single (Van Den Hurk 2007) and multiplex PCR assays (Kent and Norris 2005). Although this method is relatively simple and inexpensive, it is only practical for screening samples by class or order, or determining if a mosquito has fed on one or a very few species of interest (eg. to determine human blood index).

Direct sequencing of a portion of the host *cytb* gene extracted from the blood meal has also been used for host blood meal identification (Cupp *et al.* 2004b, Kilpatrick *et al.* 2006a, Molaei *et al.* 2006). This method uses one or more primer sets to produce *cytb* fragment(s) that may be sequenced and compared to known *cytb* sequences from databases such as GenBank (NCBI). This method is highly specific, but may not distinguish multiple sequences present in the same sample (Meece *et al.* 2005), although the use of class-specific primer sets may allow identification of mixed meals from both avian and mammalian hosts (Molaei *et al.* 2006).

A PCR-heteroduplex (HDA) assay was developed to identify host blood meals from engorged *Simulium damnosum s.l.* and *Glossina palpalis* to the species level (Boakye *et al.* 1999). This assay distinguished host DNA based on the mobility of heteroduplex molecules formed by the annealing of the amplified *cytb* product (sample) and a closely related probe (driver) sequence. This method has been used successfully to identify host DNA from blood fed

mosquitoes (Apperson *et al.* 2002, Lee *et al.* 2002, Hassan *et al.* 2003, Ngo and Kramer 2003, Cupp *et al.* 2004a). This method has been criticized as more subjective and less reproducible than other *cytb*-based molecular methods (Meece *et al.* 2005).

A fourth approach, the Terminal Restriction Fragment Length Polymorphism (TRFLP) method, has been used to distinguish host identity to the species level based on the position of restriction enzyme binding sites. To determine host identity, the Terminal Restriction Fragment (TRF) length profiles of host DNA from engorged mosquitoes collected from the field were compared with TRF length profiles from known vertebrate samples. Ngo and Kramer (2003) used a restriction enzyme assay to identify mosquito blood meals using species specific locations of restriction sites for *Bam*HI and *Pfl*MI to distinguish American crow *cytb* products from *cytb* products amplified from other passeriformes species. TRFLP assays on host *cytb* DNA have been used to identify host DNA from blood meals in *Ixodes ricinus* (Kirstein and Gray 1996), *Glossina palpalis palpalis* (Steuber *et al.* 2005), and several mosquito species (Meece *et al.* 2005, Oshaghi *et al.* 2006). Advantages of the TRFLP method include: (i) a more quantitative result than PCR-HDA, and (ii) a greater ability to distinguish patent multiple blood meals (ie. multiple blood meals from different host species) than direct sequencing or PCR-HDA (Zehner *et al.* 1998).

In addition to host species identification, molecular techniques have been developed to identify individual hosts from mosquito blood meals using DNA profiling (Coulson *et al.* 1990). The use of DNA fingerprinting techniques has been limited to identify specific human hosts of highly anthrophilic (and typically endophagic) mosquito species in a single village or household (Chow-Shaffer *et al.* 2000, Michael *et al.* 2001, De Benedictis *et al.* 2003).

1.14 Host Range of Important *Culex* Species in Louisiana

The *Cx. pipiens* complex is comprised of two species, *Cx. pipiens* and *Cx. quinquefasciatus*, as well as a number of biotypes such as *Cx. pipiens pallens* and *Cx. pipiens molestus* (Mattingly 1967, Belkin 1977). Members of the complex have a cosmopolitan distribution, with *Cx. pipiens* ubiquitous in temperate areas, and *Cx. quinquefasciatus* ubiquitous in tropical and subtropical areas, and hybrid forms where the two species overlap in North America (Barr 1957).

In Louisiana, *Cx. quinquefasciatus* is regarded as the primary enzootic vector of WNV (Godsey *et al.* 2005b, Gleiser *et al.* 2007). The contribution of *Cx. quinquefasciatus* to the enzootic amplification of WNV is dependent on the rate at which it feeds on competent reservoir hosts. Although a few North American populations of *Cx. quinquefasciatus* have been observed to be strongly ornithophagic (Bertsch and Norment 1983, Irby and Apperson 1988, Reisen *et al.* 1990), seasonal, geographical and habitat related differences in the avian feeding rate of *Cx. quinquefasciatus* have been reported, and may be greatly influenced by local host abundance. In EBRP, avian blood was detected in less than 20 % of *Cx. quinquefasciatus* females collected directly adjacent to human residences, but in over 45 % of females collected from hardwood forests (Niebylski and Meek 1992). In Florida, only 33 % of blood-fed *Cx. quinquefasciatus* collected from a sewage pond adjacent to a residential area were shown to have fed on an avian host, compared with 73% of females collected from a swamp adjacent to an agricultural area (Edman 1974). Avian species have represented a minority of hosts identified from blood-fed *Cx. quinquefasciatus* collected from, and adjacent to, residential areas in California, Arizona, and Texas (Reisen *et al.* 1990, Zinser *et al.* 2004, Molaei *et al.* 2007).

In many parts of Africa, India, Asia, Australia and South America, *Cx. quinquefaciatus* is highly anthropophillic, with observed human blood indices from 50 to 95 percent (Lee *et al.* 1954, Mboera and Takken 1999, Gomes *et al.* 2003, Van Den Hurk *et al.* 2003, Samuel *et al.* 2004), which enables *Cx. quinquefaciatus* to act as the primary vector of *Wuchereria bancrofti*, a very important human lymphatic filarial pathogen. Although the proportion of bloodmeals obtained from humans in urban habitats has been reported to be as high as 40 percent in Tucson, Arizona, and 16 to 23 percent in EBRP, Louisiana (Niebylski and Meek 1992, Zinser *et al.* 2004), most studies on the host range of North American populations of *Cx. quinquefasciatus* have reported average human feeding rates of less than 10 percent (Edman 1974, Bertsch and Norment 1983, Irby and Apperson 1988, Molaei *et al.* 2007).

Host preference studies have demonstrated a very strong affinity of *Cx. quinquefasciatus* for canine hosts (Loftin *et al.* 1997, Labarthe *et al.* 1998). In East Baton Rouge Parish, the domestic dog was identified as the most common host of *Cx. quinquefasciatus* (Niebylski and Meek 1992). Canine blood was detected in over two-thirds of blood-fed *Cx. quinquefasciatus* collected from residential neighborhoods, and approximately a third of females collected from adjacent hardwood forests. The domestic dog also has been identified as the primary host of *Cx. quinquefasciatus* in Texas (Kokernot *et al.* 1974, Molaei *et al.* 2007), and the most common mammalian host in Hawaii, southern California and Florida (Hess and Hayes 1970, Edman 1974, Reisen *et al.* 1990). The host preference for dogs may reflect the very close association of *Cx. quinquefasciatus* with suburban and urban development (Schreiber *et al.* 1989, Reisen *et al.* 1992, Savage *et al.* 2006).

Another species of *Culex* implicated as an important arbovirus vector in Louisiana is *Cx. nigripalpus.* Host range data suggest that this species may be highly opportunistic. In a study in

Florida, the proportion of females feeding on mammalian hosts ranged by site from approximately 50 to over 90 percent, depending on the local vertebrate faunal composition (Edman 1974). In an urban area in Brazil, host bloodmeals from 45.3 percent of bloodfed *Cx. nigripalpus* females reacted with anti-chicken antibodies, indicating an avian host (Gomes *et al.* 2003). In Panama, almost 90 percent of the *Cx. nigripalpus* blood meals identified by CPT were of avian origin (Christensen *et al.* 1996). In Florida, a very low rate of feeding on humans has been reported (Provost 1969), and very few host seeking *Cx. nigripalpus* females are observed in human landing collections (Bidlingmayer 1967), suggesting that humans are not a preferred host for this mosquito species.

Culex salinarius is considered a potentially important bridge vector of WNV (Turell *et al.* 2005). Evidence from host utilization studies suggests that *Cx. salinarius* is highly opportunistic. Bloodfed *Cx. salinarius,* collected from largely urban sites in Connecticut, were found to feed primarily on mammals, with the white-tailed deer (*Odocoileus virginianus*) representing more than one third of all blood meals identified by *cytb* sequencing (Molaei *et al.* 2006); less than two percent of the *Cx. salinarius* blood meals in this study were identified as human derived. In rural North Carolina, almost 90 percent of *Cx. salinarius* blood meals were of mammalian origin, with canines identified by CPT and indirect ELISA as the most important hosts (Irby and Apperson 1988). Similarly, over 90 percent of the engorged *Cx. salinarius* collected from sites adjacent to Tampa, Fl, were reported to have fed on mammalian hosts, the majority identified as lagomorphs (Edman 1974). Over three quarters of engorged *Cx. salinarius* collected from salt marsh habitats in southern Louisiana were shown to have fed on a bovine host; with only three percent identified as avian (Schaefer and Steelman 1965). In contrast, 45 percent of the engorged *Cx. salinarius* collected from urban and suburban habitats in Jefferson Parish, Louisiana, were found

to have fed on an avian host (Cupp and Stokes 1976). The remaining engorged *Cx. salinarius* collected by Cupps and Stokes (1976) contained primarily equine (17%), canine (15%) or human (5%) blood.

Culex restuans is generally regarded to be highly ornithophagic. The proportion of bloodmeals from *Cx. restuans* identified as avian have ranged from 100 percent in Massachusetts and Connecticut (Magnarelli 1977, Nasci and Edman 1981, Molaei *et al.* 2006), 94 percent in North Carolina (Irby and Apperson 1988), 80 to 87 percent in New York (Apperson *et al.* 2002, Apperson *et al.* 2004), to 52 percent in New Jersey (Apperson *et al.* 2004). Although Gingrich and Williams (2005) only detected avian blood in 10 percent of engorged *Cx. restuans* collected from Deleware, the sample size was very small (n=10). Host range studies have never been conducted on a population of *Cx. restuans* from the southern United States.

Although the vector competence of *Cx. erraticus* (Dyar and Knab) for WNV has not been evaluated, evidence of WNV infection in this species has been reported in Florida and the Tennessee Valley (Hribar *et al.* 2003, Cupp *et al.* 2007). *Cx. erraticus* is considered to be an opportunistic blood feeder that will feed readily on mammals, birds, reptiles or amphibians. Engorged females collected from rural North Carolina were identified by CPT and indirect ELISA as having fed on avian, mammalian and poikiotherm (reptilian or amphibian) hosts in nearly equal proportions (Irby and Apperson 1988). In a similar study in North Carolina, Robertson *et al.* (1993) detected mammalian blood in 49 percent, avian blood in 31 percent, and poikiotherm blood in 20 percent, of all blood fed *Cx. erraticus* collected. The host range of *Cx. erraticus* may be dominated by one vertebrate class, depending on local host abundance. In Tennessee, 86 percent of *Cx. erraticus* bloodmeals were from mammalian hosts, primarily dogs (Apperson *et al.* 2004). Host range studies in Alabama, reported that most of the bloodfed *Cx.*

erraticus collected had fed on an avian host (Cupp *et al.* 2004b). In Panama, over two-thirds of the *Cx. erraticus* blood meals identified by CPT were from reptilian or amphibian hosts, approximately 20 percent were from mammals, and less than 13 percent were from avian sources (Christensen *et al.* 1996).

The public health importance of *Cx. coronator* is unclear. Although WNV has been detected in *Cx. coronator* (CDC 2007e), the vector competence of this species for WNV has not been evaluated. Laboratory studies have shown *Cx. coronator* to be a poor vector of VEEV (Turell et al. 2000a). Few studies have attempted to determine the host range of *Cx. coronator*. This species was until recently only collected from the southern parts of Texas and New Mexico, and southeastern Arizona (Darsie and Ward 2005). The first confirmed collection of *Cx. coronator* specimens from Louisiana was in 2004 (Debboun *et al.* 2005). This species appears to prefer feeding on large mammalian hosts. Large numbers of host-seeking females have been collected from equine hosts in Mexico, Texas and New Mexico (Jones *et al.* 1977, Reyes-Villanueva *et al.* 2006), and human hosts in Belize, Brazil and Mexico (Bertram 1971, Roberts *et al.* 1981, Reyes-Villanueva *et al.* 2006). Small numbers of *Cx. coronator* have also been collected from rodent baited traps in western Texas (Easton *et al.* 1968). A sample of seven blood-fed *Cx. coronator* females collected from the southeastern region of the United States (presumably Texas) were examined by precipitin test, all reacted with rabbit antisera (Suyemoto *et al.* 1973).

1.15 Seasonal Host Utilization

A number of arboviruses of public health and veterinary importance in North America, such as Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), SLEV and WNV, are primarily maintained in an enzootic transmission cycle involving avian

reservoir hosts and ornithophagic mosquito vectors. Epidemic transmission of these disease agents to humans requires a bridge vector to feed on an infected bird, then subsequently feed on a human host. Seasonal changes in the host-utilization patterns of a competent vector species, from feeding primarily on avian reservoir hosts during the spring and early summer, to feeding on humans and other incidental hosts later in the summer, may allow a mosquito species to serve as both an enzootic vector and a bridge vector.

In Florida, a shift from feeding primarily on avian hosts in the spring and early summer, to feeding on mammalian hosts in the late summer and fall, has been described in *Cx. nigripalpus* (Edman and Taylor 1968, Edman 1974). It has been suggested that spatial-temporal changes in surface hydrology are responsible for this shift in blood feeding behavior, and may facilitate the amplification of SLEV and WNV in avian reservoir hosts (Shaman *et al.* 2005, Shaman *et al.* 2004). Extreme drought in the spring restricts feeding activity to wetland areas where avian species are the most abundant hosts. Wetting later in the summer allows *Cx. nigripalpus* to disperse from the wetland areas to more upland/open habitats where mammalian hosts are more common. This timing and magnitude of the host shift in *Cx. nigripalpus* varies considerably from year to year, and is associated with the timing and duration of the rainy season in Florida (Edman 1974).

There has been great interest in describing the seasonal patterns of host-utilization by *Cx. tarsalis*, the primary vector of Western Equine Encephalitis virus (WEEV) and WNV in many parts of the western United States and Canada. A seasonal shift in the host utilization of *Cx. tarsalis*, from feeding primarily on avian hosts in the spring and early summer, to feeding on mammalian hosts in the late summer and fall, was first documented in Kern County, California (Reeves *et al.* 1963). In subsequent study by the same authors, a significant seasonal increase in

the proportion of *Cx. tarsalis* meals from mammalian host was consistently observed over a three year period, from less than 10 percent in the spring and early summer, to a peak of over 30 percent in the late summer (Tempelis *et al.* 1965). Other studies in southern California, and studies in Colorado and Texas, have demonstrated a similar shift in the seasonal host utilization of *Cx. tarsalis* (Tempelis and Washino 1967, Tempelis *et al.* 1967, Gunstream *et al.* 1971, Hayes *et al.* 1973). However, the seasonal pattern of bloodfeeding behavior in *Cx. tarsalis* appears to vary greatly among localities. Other studies of the host range of *Cx. tarsalis* in Alberta, Kansas and southern California have failed to demonstrate a significant seasonal shift in host-utilization by *Cx. tarsalis* (Shemanchuk *et al.* 1963, Edman and Downe 1964, Lothop *et al.* 1997).

Seasonal shifts in host utilization of mosquitoes have been implicated as a factor increasing the risk of transmission of WNV to humans in the northeastern United States. The American Robin (*Turdus migratorius*), a moderately competent reservoir host for the virus (Komar *et al.* 2003a), is a preferred host of the primary enzootic vectors in this region, *Cx. pipiens* and *Cx. restuans*. In Connecticut and Maryland, a seasonal shift in *Cx. pipiens*, from feeding on the American Robin to other hosts during the late spring and summer months, has been associated with robin dispersal (Kilpatrick *et al.* 2006a, Molaei *et al.* 2006). In Maryland, a concurrent increase in the human feeding rate was associated with the decline in meals from the American Robin (Kilpatrick *et al.* 2006a). However, no significant seasonal change in the mammalian or human feeding rate was detected in *Cx. pipiens* in Connecticut (Molaei *et al.* 2006).

There has been some evidence of a seasonal shift in the feeding pattern of *Cx. quinquefasciatus*. In Mississippi, the proportion of *Cx. quinquefasciatus* blood meals acquired from avian hosts was observed to decline during the summer months and then increase in the fall

(Bertsch and Norment 1983). In Texas, a significant shift was observed in the feeding behavior of *Cx. quinquefasciatus*; the proportions of bloodmeals obtained from mammalian to avian hosts was nearly equal from March to August, but avian hosts only accounted for only 27 percent of all identified bloodmeals in females collected in September, and less than 15 percent of all identified bloodmeals in females collected during October and November (Molaei *et al.* 2007). In EBRP, Niebylski and Meek (1992) did not observe significant seasonal changes in the proportion of *Cx. quinquefasciatus* feeding on birds in hardwood forest sites. The authors did report a seasonal decline in the avian feeding rate of *Cx. quinquefasciatus* collected from ovitraps at adjacent sites in a residential area; from 30 percent in June, to 6 percent in late August and September. However, this decline in avian feeding was attributed to the removal of several large nesting boxes from the residential area in the middle of July.

Seasonal host-utilization trends have been reported for other mosquito vector species. In Jefferson Parish, Louisiana, Cupp and Stokes (1976) noted that the proportion of female *Cx. salinarius* that had fed on avian hosts was greater in the late fall and winter, though the difference was not statistically significant. In Alabama, it was noted that the proportion of *Cx. erraticus* feeding on avian hosts declined during the summer (Hassan *et al.* 2003). This decline was attributed to a seasonal reduction in nesting behavior in the Yellow-crowned Night-heron, the most common avian host of *Cx. erraticus* detected in the study. In the highly ornithophilic species *Culiseta melanura* (Coquillett), mammalian bloodmeals were only detected in females collected during the late summer and early fall in Maryland (LeDuc *et al.* 1972). It is apparent that local changes in seasonal host availability can influence the seasonal pattern of host utilization in many mosquito species.

1.16 Vertical and Venereal Transmission of WNV

A mosquito may acquire an arbovirus infection by several routes. For female mosquitoes, an arbovirus can be obtained horizontally by an oral route during blood feeding on an infective vertebrate host, or by a venereal route during copulation via seminal fluid obtained from an infected male mosquito. Many arboviruses also are transmitted vertically from an infected female mosquito to her progeny. Both venereal and vertical transmission mechanisms may enhance arbovirus survival during periods when horizontal transmission between arthropod vectors and vertebrate hosts is limited.

Although vertical and venereal transmission have been best described for mosquito-borne viruses in the family Bunyaviridae, both mechanisms have been demonstrated in a number of the Flaviviridae, including St. Louis Encephalitis virus (SLEV), Yellow Fever virus (YFV), Dengue virus (DEV), Japanese Encephalitis virus (JEV), and West Nile virus (WNV) (Rosen *et al.* 1978, Aitken *et al.* 1979, Hardy *et al.* 1980, Nayar *et al.* 1986, Rosen 1987a, Rosen *et al.* 1989, Baqar *et al.* 1993, Reisen *et al.* 2006c). Vertical transfer of bunyaviruses from an infected female mosquito to her progeny occurs when the virus infected the ovaries (transovarial transmission). In contrast, flaviviruses enter the fully developed egg during fertilization, which is considered a much less efficient form of vertical transmission (Rosen 1987a).

The first successful demonstration of vertical WNV transmission in mosquitoes was in *Ae. albopictus* parenterally infected with the Kunjin virus subtype of WNV (Tesh 1980). In this study, Kunjin virus was not vertically transmitted in *Cx. quinquefasciatus*, *Ae. vexans* or *Toxorhynchites amboinensis*. Baqar *et al.* (1993) subsequently tested vertical transmission in *Cx. tritaeniorhynchus*, *Ae. aegypti* and *Ae. albopictus* infected by intrthoracic injection with WNV. Vertical transmission was demonstrated in all three species, with significantly higher minimum

filial infection rates (MFIR) observed in *Ae. aegypti* and *Ae. albopictus*. However, Dohm *et al.* (2002b) was unable to demonstrate vertical transmission in *Ae. albopictus* intrathoracically injected with a New York strain of WNV. The MFIR of the F1 progeny of parenterally infected *Cx. pipiens* had MFIR rates between 1.4 to 2.1 per 1000 (Dohm *et al.* 2002b). Vertical transmission of North American strains of WNV has also been demonstrated in parenterally infected *Cx. quinquefasciatus*, and parenterally and orally infected *Cx. tarsalis* (Goddard *et al.* 2003, Reisen *et al.* 2006c).

The first field evidence of vertical transmission of WNV was the isolation of virus from a pool containing four male mosquitoes of the *Cx. univittatus* complex that had been collected from western Kenya (Miller *et al.* 2000). Since then, a significant amount of evidence of vertical transmission of WNV in natural vector populations in North America has been accumulated. In the northeastern United States, WNV has been detected in male *Cx. pipiens*, in nulliparous and/or overwintering female *Cx. pipiens*, and in the progeny of naturally infected, overwintering female *Cx. pipiens* (Nasci *et al.* 2001, Bugbee and Forte 2004, Farajollahi *et al*. 2005, Anderson *et al.* 2006, Anderson *et al*. 2007). However, other attempts to detect WNV in overwintering female *Cx. pipiens* in Colorado, and male *Cx. pipiens* collected from Ohio during the summer, were unsuccessful (White *et al.* 2006, Bolling *et al.* 2007). Reisen *et al.* (2006c) failed to detect WNV in diapausing female *Cx. tarsalis* collected from overwintering sites in southern California, but was able to detect virus in male *Cx. quinquefasciatus* that had been collected from gravid traps, and adult male *Cx. quinquefasciatus* reared from field collected egg rafts, during the summer months. West Nile virus also has been detected in field collected larvae of *Cx. erythrothorax* Dyar collected from Utah (Phillips and Christensen 2006).

Venereal transmission of WNV and SLEV by mosquitoes has been demonstrated experimentally (Shroyer 1990, Reisen *et al.* 2006c). Reisen *et al.* (2006c) observed venereal transfer of WNV in *Cx. tarsalis*, from parenterally infected males to uninfected females, but only a small proportion of females retained the infection for 3-5 days post-mating, and no virus was detected in the progeny. However, these were very small scale experiments; only 21 females were successfully force-mated to infected males. Larger studies will be required to determine if venereal transmission represents a dead-end pathway for WNV.

1.17 Study Objectives

An objective of this research was to obtain basic information on the role of various mosquito species in the transmission cycles of WNV in Louisiana. This was first accomplished by collecting baseline data on WNV infection rates in various mosquito species in EBRP, and then examining spatial and temporal trends in WNV infection in vector populations. The information from this study will help identify potential WNV vector species in EBRP.

The second objective of this research was to determine the host range of potentially important vectors of WNV in EBRP, and to examine seasonal patterns of host utilization in the primary enzootic vector of WNV in the southeastern United States, *Cx. quinquefasciatus*. To determine the significance of a competent vector species to the transmission of WNV, it is necessary to determine its seasonal host range. This is particularly critical when evaluating the role of potential bridge vector species in transmission of the pathogens to humans and domestic animals. Direct sequencing and a TRFLP-based assay was used to provide specific host bloodmeal identification. This information will allow us to understand the specific role of each of these species in the transmission of WNV in EBRP.

Vertical and venereal transmission pathways may be important in the maintanence of WNV during the cooler parts of the year when horizontal transmission between arthropod vectors and vertebrate hosts is limited, and may enhance epizootic and epidemic transmission during the summer months by allowing nulliparous females to become infective. The third objective of the research was to examine males of several species of mosquitoes collected from EBRP for evidence of WNV infection. This information may be useful for evaluating the potential for vertical and venereal transmission of WNV by different mosquito species in EBRP.

CHAPTER 2. WEST NILE VIRUS DETECTION IN MOSQUITOES IN EAST BATON ROUGE PARISH, LOUISIANA, FROM NOVEMBER, 2002, TO OCTOBER, 2004[*]

2.1 Introduction

The first reported activity of West Nile virus (WNV) in North America was in 1999, in New York State (Lanciotti *et al.* 1999). The virus rapidly spread to the southeastern United States, with the first reported human case of West Nile virus (WNV) in Louisiana in 2001 (Balsamo *et al.* 2003). In 2002, a total of 329 human cases were reported in LA, with 24 deaths. The economic costs of the 2002 epidemic in Louisiana were substantial, including an estimated $8.3 million for mosquito surveillance and abatement (Zohrabian *et al.* 2004).

Several ornithophillic mosquitoes in the genus *Culex* have been implicated as important enzootic vectors of WNV in different regions in the United States. In the northeastern United States, *Cx. pipiens* L. has been described as the primary vector (Andreadis *et al.* 2004, Lanciotti *et al.* 1999). *Culex tarsalis* Coquillett has been implicated as the primary vector in the western and midwestern states (Goddard *et al.* 2002). In the southeastern U.S., *Cx. quinquefasciatus* Say and Cx. *nigripalpus* Theobald are considered important WNV vectors (Godsey *et al.* 2003, Rutledge *et al.* 2003).

The first detection of WNV in mosquitoes from East Baton Rouge (EBR) Parish was in pools of *Cx. quinquefasciatus* collected in 2002 (Gleiser *et al.*2007). West Nile virus was not detected in 14 other mosquito species sampled from the parish in the same year. As of 2005, evidence of WNV infection had been reported in 60 mosquito species in the United States (CDC 2005). At least 35 of these species are present in EBR parish (Chapman and Johnson 1986). Vector competence for WNV has been demonstrated experimentally for at least 12 of these

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species (Sardelis *et al.* 2001, Goddard *et al.* 2002, Tiawsirisup *et al.* 2004, Ebel *et al.* 2005, Turell *et al.* 2005).

In the Gulf Coast region, WNV transmission may occur year-round (Blackmore *et al.* 2003, Tesh *et al.* 2004), probably involving multiple vector species. Identification of the primary enzootic, epizootic and epidemic WNV vectors is essential to develop more effective surveillance and control programs for WNV transmission in Louisiana. The purpose of this study was to identify potential vectors of WNV in East Baton Rouge parish between November 2002 and October 2004, as well as to examine the seasonal activity of WNV in mosquitoes.

2.2 Materials and Methods

Mosquitoes were collected using two sampling efforts. In the first program, a total of 27 sites was sampled in urban, suburban and agricultural habitats in East Baton Rouge Parish, Louisiana, as part of a Board of Regents (BOR) supported study. Collections were made every 2 weeks, from November 2002 to October 2004. Mosquitoes were collected using Centers for Disease Control (CDC) miniature UV-light traps (Model 512; John W. Hock Co., Gainesville, FL) baited with approximately 3kg of dry ice and CDC gravid traps (Model 1712; John W. Hock Co., Gainesville, FL), baited with 2 l of a 1.2 percent fish oil emulsion that had been prepared 4-8 days previously. During each sampling week, a single light trap was operated for 24 hours at each site at a height of 1.5 m, and then for 24 hours at a height of 3 m. A gravid trap, placed a minimum of 50 m from the light trap, also was operated concurrently both evenings at each site. Collections of insects were transported to the laboratory in a cooler containing dry ice.

For the second sampling effort, mosquitoes were collected as part of the East Baton Rouge Parish Mosquito Abatement and Rodent Control (EBRPMARC) arbovirus surveillance program. Mosquitoes were sampled from 10 of the BOR sites on alternate weeks, from February

to October 2004. An additional 68 sites in EBR parish also were sampled by EBRPMARC personnel. Mosquitoes were collected from each EBRPMARC site using one or more of the following methods: fish oil baited gravid traps, CDC miniature UV-light traps, Encephalitis Virus Surveillance (EVS) light traps (Model 2801A; BioQuip Products, Rancho Dominguez, CA), Faye Prince traps (Model 712; John W. Hock Co., Gainesville, FL), Mosquito Magnet traps (American Biophysics Corp., North Kingstown, RI), Rotator Traps (Model 512; John W. Hock Co., Gainesville, FL), backpack aspirators (Model 1412; John W. Hock Co., Gainesville, FL), chicken baited Florida Medical Entomology Laboratory (FMEL) traps (Rutledge *et al.* 2003), or chicken baited sentinel box traps.

Mosquitoes were sorted on a chill table, and females were pooled by species, collection site, collection method, and by date of collection. The sorted female mosquitoes were then placed into sterile, 2 ml Costar® microcentrifuge tubes (Corning Inc., Corning, NY) containing a maximum of 50 females per pool. All WNV detection assays were performed at the LSU Veterinary School Diagnostic Laboratory. For all three detection WNV methods used in the current study, a sterile copper-coated BB and 1.5 ml of BA-1 diluent (Hanks M-199 salts, 3.3% bovine serum albumin, 0.034% sodium bicarbonate, 100U / ml penicillin, 0.1 mg / ml streptomycin, 2.5 mg / l amphotericin B, 0.05M TRIS buffer ph 7.4) was added to each pool. Mosquitoes were homogenized in a Retsch MM300 mixer mill (Qiagen Ltd), for 5 minutes at 25Hz, and then centrifuged in a refrigerated centrifuge for 6 minutes at 6200 rpm.

Mosquitoes collected in the BOR study were tested for WNV using the virus isolation method. Plaque assays were conducted by re-centrifuging 0.5 ml of the supernatant from each sample at 6200 rpm for 10 minutes. Two wells of a tissue culture plate (Costar, Corning, NY) containing confluent Vero cell monolayers were inoculated with 10 µl of supernatant from each

mosquito pool. After 96 h and 120 h of incubation at 37°C in a 5% CO_2 atmosphere, cultures were examined for cytopathic effects. Culture supernatant from pools exhibiting cytopathic effects was passaged a second time on Vero cells to confirm infection. The presence of WN, EEE and/or SLE virus in cultures exhibiting cytopathic effects was confirmed by RT-PCR.

Samples collected by EBRPMARC for arbovirus surveillance were tested by the VecTest assay in 2003 and by RT-PCR in 2004. The VecTest WN/SLE antigen panel assay (Medical Analysis Systems, Camarillo, CA) was performed on EBRPMARC surveillance samples as described by Nasci *et al.* (2002). Briefly, 125 µl of each mosquito homogenate was mixed 1:1 with grinding solution provided with the assay kit, and incubated with a VecTest test strip at room temperature for 15 minutes.

For the RT-PCR, extraction of RNA was performed using the Qiagen QIAamp® Virus Biorobot® 9604 Kit. A 220 µl volume of cleared mosquito homogenate was mixed with 240 µl of AL buffer and 40 µl of protease, and incubated at 60 ºC for 10 minutes. After the addition of 265 µl of 100 % ethanol, samples were transferred to a QIAamp® 96 plate (Qiagen) and washed three times. A volume of 86 µl of elution buffer was used to elute RNA from the Qiagen columns. Wash and elution steps were performed using a 5,796 x g centrifugation speed. Eluted RNA was stored at -20ºC until testing.

The QuantiTect Probe PCR Kit (Qiagen) was used to perform real-time RT-PCR. Each 15 µl reaction contained 15 pmol of each primer, 3 pmol of probe, and 5 µl of eluted RNA. The 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. Primers and probes were developed

specifically for the NY99 strain, flamingo 382-99 (National Center for biotechnology Information, 2005). The samples were subjected to 45 cycles of amplification in an ABI 7900HT real time PCR instrument (Applied Biosystems, Foster City, CA). The following cycling conditions were used: 1 cycle of 48 ºC for 30 minutes and 95 ºC for 10 minutes, followed by 45 cycles of 95 ºC for 15 seconds, and 60 ºC for 1 minute. Results were given as cycle threshold (CT) units, which is the cycle number at which fluorescence of the probes is significantly greater than the background fluorescence. The Ct value of each sample is inversely related with the amount of template present; lower values indicate a greater amount of viral RNA in the sample. Pools were considered positive when CT units were less than 37. Infection rates were calculated using the maximum likelihood estimation (MLE) method (Biggerstaff 2003). The proportions of infected pools collected at each height in the BOR study were compared by a two-tailed Fisher's Exact Test (Quickcalcs, GraphPad Software, Inc., San Diego, CA).

2.3 Results

In the BOR study, 244,374 female mosquitoes of 36 species were collected (Table 2.1); the majority (57.7%) was from CDC UV-light traps. West Nile virus was isolated from pools of at least 17 (47.2%) mosquito species (Table 2.2). About 0.6 percent (80/14,236) (95% Confidence Interval (CI): 0.5-0.7) of all submitted pools of female mosquitoes tested positive for WNV by viral isolation. In 2003, 68 mosquito pools tested positive for WNV; the majority (81%; 95% CI: 69.2 – 88.5) of positive pools contained mosquitoes collected in March and April (Table 2.3). After April 2003, most (76.9%) of the WNV positive pools contained *Culex* mosquitoes. In 2004, the only isolations of WNV were from 12 pools of *Culex* mosquitoes.

In the BOR-collected samples, the proportion of pools of *Cx. salinarius* Coquillett collected by light trap at the 3m height that were positive for WNV (5/292) was significantly

Table 2. 1. Total mosquitoes collected in East Baton Rouge Parish for WNV detection by virus isolation method, November, 2002, to October, 2004

Species	No. Pools Tested	No. of Females Tested			
		Light Trap height		Gravid Trap	Total (% of Total Catch)
		1.5 m	3 m		
Culex quinquefasciatus	3336	1661	2205	96804	100670 (41.2)
Aedes vexans	2168	37090	27002	141	64233 (26.3)
Cx. nigripalpus	606	6734	7080	151	13965 (5.7)
Psorophora ferox	483	4825	5582	36	10443 (4.3)
Cx. erraticus	709	3404	2623	94	6121 (2.5)
Anopheles crucians	773	3168	2086	9	5263 (2.2)
Unidentified *Culex* species	529	619	594	4038	5251 (2.1)
Cx. salinarius	727	2937	1804	126	4867 (2.0)
Ae. albopictus	809	2414	1159	1032	4605 (1.9)
Ochlerotatus dupreei	160	1869	1427	1	3297 (1.3)
Oc. atlanticus / tormentor	255	1964	922	30	2916 (1.2)
Oc. infirmatus (Dyar and Knab)	238	1672	1138	7	2817 (1.2)
Unidentified *Psorophora* species	153	1365	750	0	2115 (0.9)
Oc. canadensis	132	1151	682	15	1848 (0.8)
Unidentified *Ochlerotatus* species	164	1581	401	19	2001 (0.8)
Oc. triseriatus (Say)	314	1270	578	18	1866 (0.8)
Uranotaenia sapphirina (Osten Sacken)	298	1097	973	5	2075 (0.8)
Cx. restuans	382	541	356	722	1619 (0.7)
An. quadrimaculatus (Say)	414	878	624	4	1506 (0.6)
Mansonia tittilans	269	836	602	11	1449 (0.6)
Ps. columbiae	178	762	540	3	1305 (0.5)
Cx. coronator	74	529	184	4	717 (0.3)
Coquillettidia perturbans	205	450	361	5	816 (0.3)
Culiseta inornata	191	287	233	6	526 (0.2)
Oc. fulvus pallens (Ross)	117	367	237	1	605 (0.2)
Ur lowii	126	275	239	6	520 (0.2)
An. punctipennis (Say)	116	158	104	2	264 (0.1)
Ps. howardii	87	126	81	0	207 (0.1)
Other[1]	223	278	194	15	487 (0.2)
Total	14236	80308	60761	103305	244374

[1] Includes *Cu. melanura* (Coquillett), *Cx. tarsalis*, *Cx. territans* (Walker), *Oc. mitchellae* (Dyar), *Oc. taeniorhynchus* (Wiedemann), *Oc. trivittatus* (Coquillett), unidentified *Orthopodomyia* species, *Or. signifera* (Coquillett), *Ps. ciliata* (Fabricius), *Ps. cyanescens* (Coquillett), *Ps. horrida* (Dyar & Knab), *Ps. mathesoni* (Belkin & Heinemann) and unidentified *Uranotaenia* species.

Table 2. 2. West Nile virus isolations from mosquitoes collected in East Baton Rouge Parish for Board of Regents Project, November, 2002, to October, 2004

Year	Species	No. Infected Pools	No. Pools Tested	No. Females Tested	Infection Rate (95% Confidence Intervals)[1]
2003	*Culex restuans*	10	171	647	15.7 (8.3 – 27.3)
	Cx. salinarius	5	388	2398	2.1 (0.8 – 4.6)
	Cx. erraticus	4	388	2596	1.5 (0.5 – 3.7)
	Cx. nigripalpus	4	503	13225	0.3 (0.1 – 0.7)
	Cx. quinquefasciatus	11	1745	52436	0.2 (0.1 – 0.3)
	Ochlerotatus canadensis	5	49	568	9.3 (3.6 – 20.5)
	Oc. triseriatus	2	130	456	4.3 (0.8 – 13.8)
	Oc. atlanticus / tormentor	2	109	940	2.1 (0.4 – 6.8)
	Aedes albopictus	1	399	1851	0.5 (<0.1 – 2.6)
	Ae. vexans	9	917	23183	0.4 (0.2 – 0.7)
	Anopheles punctipennis	1	43	68	14.5 (0.9 – 67.3)
	An. crucians	8	375	1595	5.1 (2.4 – 9.7)
	An. quadrimaculatus	2	204	597	3.3 (0.6 – 10.8)
	Coquillettidia perturbans	1	92	270	3.7 (0.2 – 17.8)
	Psorophora howardii	1	31	71	13.8 (0.8 – 64.0)
	Ps. ferox	1	174	2173	0.5 (<0.1 – 2.2)
	Uranotaenia lowii	1	82	372	2.7 (0.2 – 12.7)
2004	unidentified *Culex* species	3	276	3576	0.8 (0.2 – 2.3)
	Cx. nigripalpus	1	98	735	1.3 (0.1 – 6.4)
	Cx. quinquefasciatus	8	1343	44415	0.2 (0.1 – 0.3)

[1] Estimated number of infected females per 1000; determined by Maximum Likelihood Estimation.

higher (p=0.014) than the proportion of pools of *Cx. salinarius* collected by light trap at the lower height (0/392). No significant differences in infection rates of pools of other species were detected between the two trap heights.

Almost all of the female *Cx. quinquefasciatus* specimens submitted for WNV testing by EBRPMARC were collected using fish oil baited gravid traps (94.7%; Table 2.4). Collections from light traps (EVS, CDC light traps and rotator traps) accounted for the greatest proportion of female *Aedes vexans* (Meigen) (54.4%), *Cx. nigripalpus* (64.3%), *Cx. salinarius* (84.8%), *Mansonia tittalins* (Walker) (89.2%), *Anopheles crucians* Wiedemann (77.1%), *Psorophora*

Table 2. 3. Seasonal WNV activity in mosquitoes collected for Board of Regents Project, and human WNV cases, in East Baton Rouge Parish.

Year	Season	No. Human Cases	Species	No. Females Tested	No. Pools WNV + (%)
2003	March / April	0	*Aedes vexans*	8832	9 (3.4)
			Culex quinquefaciatus	853	7 (6.7)
			Cx. salinarius	580	3 (3.1)
			Ochlerotatus canadensis	557	5 (10.9)
			Anopheles crucians	547	8 (8.8)
			Cx. restuans	254	10 (16.4)
			Cx. erraticus	212	4 (7.7)
			Oc. atlanticus/tormentor	125	2 (8.7)
			An. quadrimaculatus	112	2 (7.1)
			Coquillettidia perturbans	84	1 (5.6)
			Oc. triseriatus	79	2 (7.1)
			An. puncipennis	28	1 (7.7)
			Psorophora ferox	28	1 (6.3)
	May / June	0	*Cx. quinquefaciatus*	18442	1 (0.2)
	July / August	2	*Cx. quinquefaciatus*	11585	2 (0.5)
			Ae. albopictus	1100	1 (0.6)
			Uranotaenia lowii	160	1 (2.6)
	September / October	2	*Cx. quinquefaciatus*	10374	1 (0.3)
			Cx. nigripalpus	7346	3 (1.2)
			Cx. salinarius	358	2 (3.4)
			Ps. howardii	12	1 (10.0)
	November / December	0	*Cx. nigripalpus*	5158	1 (5.3)
2004	July / August	18	*Cx. quinquefaciatus*	16259	6 (1.3)
			Cx. spp.	1449	3 (3.1)
			Cx. nigripalpus	51	1 (3.0)
	September / October	11	*Cx. quinquefaciatus*	12050	2 (0.7)

columbiae (Dyar and Knab) (72.1%), *Coquillettidia perturbans* (Walker) (74.9%), and *Cx. coronator* Dyar and Knab (92.4%) specimens tested for WNV antigens or WNV RNA. The majority of female *Ps. ferox* (Humboldt) (82.8%), *Cx. erraticus* (Dyar and Knab) (69.7%), *Ae. albopictus* (Skuse) (65.7%), *Oc. atlanticus/tormentor* (88.0%), and *Oc. dupreei* (Coquillett) (88.3%) specimens were collected using Faye Prince Traps. Most of the *Oc. canadensis*

Table 2. 4. Total females mosquitoes collected in East Baton Rouge Parish by EBRPMARC from November 2002 to December 2003, and February to October 2004.

	Collection Method[1]							
Species	Gravid	L.T.	F.P.	M.M.	Rot.	C.B.T.	Other[2]	Total
Cx. quinquefasciatus	241419	2274	2133	236	592	1445	6857	254956
Ae. vexans	31	3980	2322	4502	4897	11	582	16325
Ps. ferox	9	631	5964	288	131	5	171	7199
Cx. erraticus	49	381	2717	155	570	17	9	3898
Ae. albopictus	684	212	2376	62	46	25	213	3618
Oc. atlanticus/tormentor	21	101	2093	19	135	6	4	2379
Cx. nigripalpus	57	744	116	474	434	6	0	1831
Cx. salinarius	4	910	56	149	316	0	11	1446
Oc. canadensis	0	179	42	1131	47	12	0	1411
Cx. restuans	329	354	11	71	74	11	148	998
Ma. titallins	1	744	20	76	64	1	0	906
An. crucians	2	434	127	76	256	0	0	895
Ps. columbiae	1	28	86	124	533	0	6	778
An. quadrimaculatus	9	64	146	183	21	27	7	457
Oc. infirmatus	0	75	206	83	72	2	0	438
Oc. triseriatus	8	36	151	81	43	2	52	373
Oc. dupreei	0	4	272	0	32	0	0	308
Cq. perturbans	2	151	11	39	10	2	0	215
Cu. inornata	3	38	0	94	22	0	21	178
Cx. coronator	0	0	8	0	97	0	0	105
Other species[3]	4	98	89	46	112	3	5	357
Total	242633	11438	18946	7889	8504	1575	8086	299071

[1] Gravid = Fish-oil baited CDC Gravid Trap, L.T. = Light Trap (CDC light trap or EVS trap), F.P. = Faye Prince Trap, M.M. = Mosquito Magnet Trap, Rot. = Rotator Trap, C.B.T. = Chicken-baited Trap (FMEL or Sentinel Box Trap), Asp. = Backpack Aspirator.
[2] Includes Backpack Aspirator, Ovitrap, Resting Box, New Jersey Light Trap, and collection method unspecified.
[3] Includes *Oc. fulvus pallens*, *Ps. howardii*, *Ur. sapphirina*, *An. punctipennis*, *An. pseudopunctipennis* Theobald, *Oc. trivittatus*, *Or. signifera*, *Ps. ciliata*, *Ps. cyanescens*, *Ps. mathesoni*, and *Ur. lowii*.

(Theobald) (80.2%), and *Culiseta inornata* (Williston) (52.8%), and *An. punctipennis* (Say) (54.9%) specimens were collected using the Mosquito Magnet Trap.

From November, 2002, to December, 2003, a total of 131,896 mosquitoes were collected by EBRPMARC and tested for WNV antigens using the VecTest assay (Table 2.5). West Nile

virus antigens were detected in 9 (33.3%) mosquito species. Mosquitoes in 78.9 percent of the pools positive for WNV were collected in fish oil baited gravid traps; all of them were *Cx. quinquefasciatus*. Estimated infection rates in female *Cx. quinquefasciatus* collected by fish-oil baited gravid traps from January to December, 2003, were higher in the EBRPMARC samples tested by VecTest (0.6; 95% CI: 0.4 - 0.7) than in the samples collected in 2003 and tested by viral isolation as part of the BOR study (0.2; 95% CI: 0.1 - 0.3). In *Cx. restuans* Theobald, lower estimated infection rates were observed in the EBRPMARC samples than in the 2003 BOR samples. Estimated infection rates in *Cx. salinarius, Cx. nigripalpus, Ae. vexans, An. crucians, Ps. howardii* Coquillett, and *Ps. ferox* were similar between BOR collected and EBRPMARC collected samples.

In 2004, WNV was detected by RT-PCR in 14 (50%) of the mosquito species collected by EBRPMARC from February to October, 2004, and tested for WNV RNA by RT-PCR (Table 2.6). Female *Cx. quinquefasciatus* represented 81.2 percent of the 167,175 mosquitoes collected, and accounted for 95.8 percent (475/496) of the pools positive for WNV RNA. The estimated infection rate for female *Cx. quinquefasciatus* collected from February to October using gravid traps was significantly higher in the EBRPMARC collected samples (3.8; 95% CI: 3.5 – 4.2) than in the samples tested by virus isolation as part of the BOR study (0.2; 95% CI: 0.1 - 0.4). Evidence of WNV infection was detected in 12 species in the EBRPMARC collected samples that were not positive for WNV in the BOR collected samples in 2004.

2.4 Discussion

In 2003, the proportion of pools sampled that tested positive for WNV in the BOR collected samples was more than half of the proportion of pools sampled that tested positive for WNV in the EBRPMARC collected samples. In 2004, the proportion of BOR pools that tested

Table 2. 5. West Nile virus RNA detection by VecTest in mosquitoes collected from East Baton Rouge Parish by EBRPMARC, November, 2002, to December, 2003

Species	No. Pools WNV+	No. Pools Tested	No. Females Tested	WNV Infection Rate (95% Confidence Intervals)[1]
Culex restuans	1	60	632	1.6 (0.1 – 7.4)
Cx. nigripalpus	1	167	1766	0.6 (<0.1 – 2.7)
Cx. quinquefasciatus	65	3270	119250	0.6 (0.4 - 0.7)
Cx. salinarius	1	128	702	1.4 (0.1 – 6.8)
Cx. erraticus		143	643	
Ochlerotatus infirmatus	1	15	83	10.1 (0.7 – 46.6)
Oc. atlanticus / tormentor		38	135	
Oc. triseriatus		43	60	
Oc. canadensis		5	10	
Oc. fulvus pallens		4	7	
Oc. trivittatus		1	1	
Aedes vexans	3	312	5275	0.6 (0.2 -1.5)
Ae. albopictus		184	663	
Anopheles crucians	1	75	390	2.5 (0.2 – 12.0)
An. quadrimaculatus		54	88	
An. punctipennis		4	9	
Coquillettidia perturbans		50	145	
Mansonia tittilans		71	877	
Psorophora howardii	1	19	50	19.5 (1.2 – 90.2)
Ps. ferox	2	79	737	2.7 (0.5 – 8.7)
Ps. columbiae		54	143	
Ps. ciliata		5	6	
Ps. cyanescens		3	8	
Uranotaenia sapphirina		20	63	
Ur. lowii		2	4	
Culiseta inornata		41	147	
Orthopodomyia signifera		2	2	
Total	76	4849	131896	

[1] Estimated number of infected females per 1000; determined by Maximum Likelihood Estimation.

positive for WNV was less than 50-fold lower than the proportion of EBRPMARC collected pools. This may have been partially due to the sensitivity of the detection methods used. The viral isolation method used to detect WNV infection in the BOR collected samples only detects the presence of infectious virus. A significantly higher detection threshold for WNV with the cell culture assay has been reported when compared with RT-PCR (Nasci *et al.* 2002). Additionally,

Table 2. 6. West Nile virus RNA detection by RT-PCR in mosquitoes collected from East Baton Rouge Parish by EBRPMARC, February to October, 2004

Species	No. Pools WNV+	No. Pools Tested	No. Females Tested	WNV Infection Rate (95% Confidence Intervals)[1]
Culex coronator	1	19	105	8.3 (0.5 – 41.0)
Cx. quinquefasciatus	475	3392	135706	3.8 (3.4 – 4.1)
Cx. salinarius	1	80	744	1.3 (0.1 – 6.6)
Cx. erraticus	2	187	3255	0.6 (0.1 – 2.0)
Cx. restuans	1	72	366	2.6 (0.2 – 12.7)
Cx. nigripalpus		8	65	
Ochlerotatus fulvus pallens	2	13	87	24.0 (4.4 – 77.9)
Oc. triseriatus	1	75	313	3.2 (0.2 – 15.2)
Oc. atlanticus / tormentor	3	89	2244	1.4 (0.4 – 3.6)
Oc. canadensis	1	76	1401	0.7 (<0.1 – 3.4)
Oc. infirmatus		36	355	
Oc. dupreei		19	308	
Aedes albopictus	3	204	2955	1.0 (0.3 – 2.7)
Ae. vexans	1	402	11050	0.1 (<0.1 – 0.4)
Anopheles quadrimaculatus	1	54	369	2.6 (0.2 – 12.4)
An. crucians		81	505	
An. punctipennis		27	42	
An. pseudopunctipennis		1	3	
Coquillettidia perturbans		20	70	
Mansonia tittilans		6	29	
Psorophora howardii	1	3	16	38.8 (4.1 – 208.8)
Ps. ferox	3	205	6462	0.5 (0.1 – 1.3)
Ps. columbiae		43	635	
Ps. ciliata		10	29	
Ps. mathesoni		3	23	
Uranotaenia lowii		1	2	
Culiseta inornata		15	31	
Orthopodomyia signifera		1	5	
Total	496	5142	167175	

[1] Estimated number of infected females per 1000; determined by Maximum Likelihood Estimation.

the sampling strategies employed by EBRPMARC favored the collection of gravid *Cx. quinquefasciatus,* and focused on sites where WNV activity had recently been detected in mosquitoes, birds, or humans.

Mosquitoes of the species *Cx. quinquefasciatus* are very abundant and widespread in urban areas, and are regarded as the most important enzootic vectors of WNV in Louisiana (Godsey *et al.* 2005b, Gleiser *et al.* 2007). In 2003, West Nile virus antigens were detected by VecTest in 65 pools containing *Cx. quinquefasciatus*. In 2004, 475 pools containing *Cx. quinquefasciatus* were positive for WNV RNA. In the BOR-collected samples, WNV was isolated from 11 pools containing *Cx. quinquefasciatus* collected in 2003. In 2004, WNV was isolated from eight pools containing *Cx. quinquefasciatus*, and three pools containing *Culex* females collected from gravid traps in July and August. Although the unidentified *Culex* were too damaged to identify to species, they were most likely *Cx. quinquefasciatus*; the only morphologically similar *Culex* species collected in the summer in EBR parish, *Cx. salinarius* and *Cx. coronator* Dyar and Knab, represented a very small fraction of gravid trap samples (≤0.1%). Overall, *Cx. quinquefasciatus* represented only about one fifth of all BOR-collected mosquito pools submitted for WNV testing in 2004 but represented at least two thirds of all WNV isolations.

West Nile virus was detected by viral isolation in five pools of *Cx. nigripalpus* collected in the late summer and autumn of 2003 and 2004, and ten pools of *Cx. restuans* collected in the early spring of 2003. Peak *Cx. restuans* collections were in March, in 2003, and February, in 2004, and host-seeking *Cx. nigripalpus* females were most abundant from September to November in both years (data not shown). These two species may be important enzootic vectors during the late and early parts of the transmission season. *Culex quinquefasciatus* and *Cx. nigripalpus* may also serve as bridge vectors when seasonal changes in bird density cause species which normally prefer bird hosts to feed more frequently on mammalian hosts. A mid-summer shift from feeding predominantly on birds to feeding predominantly on mammalian hosts has

been observed in *Cx. nigripalpus* in Florida (Edman and Taylor 1968) and *Cx. quinquefasciatus* in Mississippi (Bertsch and Norment 1983).

West Nile virus was detected by all three methods in seven pools of *Cx. salinarius* and four pools of *Ae. albopictus* (Skuse). These two species have been implicated as important bridge vectors of WNV (Turell *et al.* 2005). West Nile virus also was isolated from a single pool of *Uranotaenia lowii* Theobald collected in August, 2003. This represents the first reported evidence of WNV infection in this species.

A significantly greater proportion of pools of *Cx. salinarius* females collected at the higher trap height (3m) were positive for WNV. In Connecticut, WNV infection rates in pools of this species collected by light traps placed at the canopy level (≈7.6m trap height) were significantly greater than in pools collected at a 1.5m trap height (Anderson *et al.* 2004). A similar difference in the frequency of WNV infection in pools of *Cx. pipiens* has been attributed to differences in physiological age structure between females collected in traps placed at ground and canopy levels (Anderson *et al.* 2006). However Drummond *et al.* (2006) failed to detect significant differences in parity rates between females collected in light traps placed at ground (≈1m) and females collected in light traps placed in the canopy (≈6-7m), in both *Cx. pipiens* and *Cx. restuans*.

The numbers of human cases of WNV reported in EBR were 4 in 2003 and 29 in 2004. In contrast, the percentage of total pools tested by viral isolation that were positive for WNV was significantly lower ($p<0.001$) in 2004 (0.2%) than in 2003 (0.9%). The onset date of the first human case was in July in both years, with the greatest number of cases reported in August. In 2003, 6.1 percent (55/908) of the pools containing mosquitoes collected in March and April were positive for WNV by virus isolation. Only 0.2 percent (9/3666) of pools containing mosquitoes

collected from May to September were positive for WNV by virus isolation. In 2004, no WNV positive pools were collected before July, and 1.2 percent (6/557) of pools containing mosquitoes collected in August were positive for WNV by virus isolation.

This study provides additional evidence that WNV transmission in East Baton Rouge Parish is primarily maintained by *Cx. quinquefasciatus*. In both years, significant WNV activity in this species was consistently detected from July through September, when the majority of human exposure to WNV was occurring. In 2004, peak numbers of pools containing *Cx. quinquefasciatus* testing positive for WNV by viral isolation and RT-PCR immediately preceded the peak in onset of human disease. This temporal association between human exposure and WNV infection in the vector was not observed in any other mosquito species. The seasonal activity and abundance, host feeding behavior, and susceptibility to infection with WNV of *Cx. quinquefasciatus* are compatible with a primary enzootic vector role, and may allow this species to serve as a bridge vector. Surveillance should be focused on monitoring infection in this species. Additional surveillance efforts to monitor infection in suspected bridge vector species, such as *Cx. salinarius* and *Ae. albopictus*, may also be important for monitoring human risk of exposure to WNV.

CHAPTER 3. HOST-FEEDING PATTERNS IN *CULEX* SPECIES

3.1 Introduction

The seasonal host range of mosquito species that are competent WNV vectors can determine the role they play in the enzootic and epidemic transmission of West Nile virus (WNV). Host range studies are particularly critical when evaluating the role of potential bridge vector species in transmission of pathogens to humans and domestic animals. To serve as a bridge vector, a mosquito must first feed on a reservoir host capable of developing a sufficient viremia for infection of the vector (ie. avian host), then feed on a human after completion of the extrinsic incubation period.

Host range studies also are used to evaluate the role of a vector species in enzootic transmission by determining the frequency of blood feeding on competent reservoir host species. Although WNV, WNV RNA or WNV neutralizing antibodies have been detected in over 250 avian species (USGS National Wildlife Health Center 2005), only a few bird species have been demonstrated to develop sufficiently high circulating viral titers to infect competent vector species, and to maintain the high viremia for a long enough period of time so that a significant number of vectors feeding on it are exposed to the virus (Langevin *et al.* 2001, Swayne *et al.* 2001, Komar *et al.* 2003a).

Temporal changes in host feeding patterns may also be important criteria when evaluating vector competence. Seasonal shifts in host feeding, from feeding mostly on avian hosts in the spring and early summer, to increasing mammalian feeding in the late summer and fall, have been described for *Culex nigripalpus* Theobald (Edman and Taylor 1968, Edman 1974), *Cx. tarsalis* Coquillett (Tempelis *et al.* 1965) and *Cx. quinquefasciatus* Say (Bertsch and Norment 1983). Changes in the host selection of primarily ornithophilic enzootic vector species

may allow rapid amplification of the pathogen in avian reservoir hosts in the spring and early summer, then increase the risk of arbovirus exposure to humans and other mammals later in the transmission season.

Specific host bloodmeal identification is an important tool for determining host range and seasonal host utilization. A variety of immunological methods have been used to detect host-specific components of bloodmeals (Bull and King 1923, Tempelis and Lofy 1963, Crans 1969, Burkot *et al.* 1981, Edrissian *et al.* 1985, Service *et al.* 1986). These methods require the production of antibodies to a wide range of potential hosts, and cross-reactivity between host antigens prohibits identification to the species level. A number of molecular-based assays recently have been developed which allow for species-specific identification of mosquito bloodmeals based on sequence polymorphisms of the cytochrome B (*cytb*) gene (Ngo and Kramer 2003, Meece *et al.* 2005, Molaei *et al*. 2006). Two of these methods, the Terminal Restriction Fragment Length Polymorphism assay and direct sequencing, were used for species-specific identification of mosquito bloodmeals in the current study.

In North America, the most important enzootic vectors of WNV, and many of the suspected bridge vectors, are mosquitoes of species in the genus *Culex* (Turell et al. 2005). The primary enzootic vector of WNV in Louisiana is *Cx. quinquefasciatus* (Godsey *et al.* 2005b, Gleiser *et al.* 2007). High densities of *Cx. quinquefasciatus* can develop in EBR Parish during the warmer months of the year, and the proportion of pools collected from gravid traps that test positive for WNV can exceed 50% during peak transmission (Chapter 2, data not shown). Vector competence studies suggest that *Cx. quinquefasciatus* is a moderately efficient vector of WNV (Sardelis *et al.* 2001, Goddard *et al.* 2002). However, its role as a potential bridge vector of WNV to human and equine hosts is not clear. *Culex quinquefasciatus* is regarded as an

opportunistic blood feeder. Seasonal, geographical and habitat related differences in host feeding behavior have been reported, and may be greatly influenced by local host abundance.

From 2002 to 2004, WNV was detected in at least 19 mosquito species collected from East Baton Rouge Parish (EBRP), including six species of *Culex* (Table 2.2, Table 2.4). Although laboratory tests have implicated some of these species as competent vectors of WNV (Turell *et al.* 2005, Tiawsirisup *et al.* 2004, Goddard *et al.* 2002, Sardelis *et al.* 2001), many are known to exhibit a broad host preference in the field. To understand the specific role each of these species in the transmission of WNV in southern Louisiana, further studies are required to determine their host range. The purpose of this study was to determine the host ranges of *Cx. coronator* Dyar and Knab, *Cx. salinarius* Coquillett, *Cx. nigripalpus* and *Cx. quinquefasciatus* in EBRP. The seasonal patterns of host utilization of *Cx. nigripalpus* and *Cx. quinquefasciatus* also were examined.

3.2 Materials and Methods

Blood-fed mosquitoes were collected from 15 sites in EBRP, 2 sites in East Feliciana Parish (EFP) and 1 site in Iberville Parish (IP) (Table 3.1, see Appendix B). Thirteen trapping sites were within 100 m of a residence and designated as 'residential'. The 13 sites designated as 'wooded' had 50 % or more of the area within a 100 m radius at least partially enclosed by forest canopy. Two of the trapping sites were located in equestrian parks, and two sites were located adjacent (within 20 m) to a permanent, freshwater swamp.

The first group of sampling locations was comprised of five sites in EBRP that were sampled over two consecutive 24 hour periods, every two weeks, from November, 2002, to October, 2004. In the second group, four EBR parish sites, the two East Feliciana parish sites, and the Iberville parish site were sampled over two consecutive 24 hour periods every two

weeks, on alternate weeks to the first sampling group, from March to November, in 2003. Collections also were made at these locations in 2004 using the same sampling regime, except for the two East Feliciana parish sites which were only sampled in 2003. The third group of locations included six sites in EBR parish that were sampled using both regimes (ie. sampled every week from March to November in 2003 and 2004, and sampled every two weeks from November, 2002, to February, 2003, and December, 2003, to February, 2004).

At each site, blood-fed mosquitoes were collected using a Centers for Disease Control (CDC) miniature UV-light trap (Model 512; John W. Hock Co., Gainesville, FL) baited with approximately 3 kg of dry ice, and a CDC gravid trap (Model 1712; John W. Hock Co., Gainesville, FL), baited with 2 liters of a 1.2 % fish oil emulsion that had been prepared 4-8 days previously. Collections of insects were transported to the laboratory in a cooler containing dry ice.

Blood-fed females were removed from trap samples on a chill table and identified to species. All females were stored at -80ºC until processed. The abdomen of each blood-fed female was removed. A four point scale (Fig. 3.1) was developed to rank the relative size of the bloodmeal, from a trace bloodmeal, barely visible under low power magnification (size I), to a full engorement (size IV).

DNA was extracted from the abdomens of blood-fed mosquitoes and from tissue samples of reference vertebrate species using the QIAamp® DNA Mini Kit and the protocols of the manufacturer for extraction of DNA from tissue (Qiagen, Valencia, CA), except that for the mosquito abdomens, 50 µl rather than 200 µl of AE buffer was used per sample to elute the extracted DNA from each QIAamp spin column. DNA extracted from field collected mosquitoes and reference vertebrate tissue samples was amplified using the primer BM1 (5’-CCC CTC

Table 3. 1. Sites sampled for blood-fed mosquitoes using CDC UV-light traps and CDC gravid traps.

Parish	Site	Latitude	Longitude	Odd Week[1]	Even Week[2]	Site Description
East Baton Rouge	Lee High School	30.4042N	-91.1513W	X	X	residential, swamp, wooded
	Farr Park	30.3856N	-91.2043W	X	X	equestrian park
	Pecue Lane	30.3805N	-91.0408W	X	X	residential, wooded
	Oneal Lane	30.4332N	-91.0074W	X	X	residential, wooded
	Emmet Bourgeois Road	30.4288N	-91.0734W	X	X	residential
	Greenwell Springs Road	30.4931N	-91.0836W	X	X	residential, swamp, wooded
	City Park	30.4328N	-91.1703W	X		wooded
	Highland Road	30.3496N	-91.0679W	X		residential, wooded
	Hoo Shoo Too Road	30.3511N	-90.9402W	X		residential, wooded
	Lazy B Stables	30.4137N	-91.0017W	X		equestrian park
	Strain Road	30.4568N	-90.9995W	X		residential, wooded
	Ednie Lane	30.5678N	-90.9870W		X	residential, wooded
	Denham Road	30.5829N	-91.0335W		X	residential, wooded
	Blackwater Road	30.5717N	-91.0704W		X	residential, wooded
	Greenwood Park	30.5688N	-91.1664W		X	wooded
Iberville	St. Gabriel Research Stn.	30.2583N	-91.1014W		X	wooded
East Feliciana	Clinton	30.7789N	-91.0658W		X[3]	residential, wooded
	Slaughter	30.7172N	-91.1414W		X[3]	residential

[1] Sampled every two weeks, from 08 November, 2002, to 21 October, 2004, on odd numbered weeks,

[2] Sampled every two weeks, from 19 March to 14 November, 2003, and 17 March to 25 November, 2004, on even numbered weeks counting from the week of 08 November, 2002.

[3] Not sampled in 2004.

AGA ATG ATA TTT GTC CTC A), labeled at the 5' end with 6-carboxyflourescein (6-FAM), and the primer BM2 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA), labeled at the 5' end with 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX) (Integrated DNA Technologies, Coralville, IA). Control DNA, for the restriction digest and terminal fragment length measurement, was amplified using unlabelled BM1 and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) labeled BM2. PCR reactions were prepared using the HotStarTaq Master Mix Kit (Qiagen). PCR reactions were performed in Fisherbrand Thin-Wall PCR Plates (Fisher Scientific, Pittsburgh, PA). Each 30 µl PCR reaction contained 15 µl of Hotstart Master Mix, 0.25 µl of each primer, 9.5 µl of dH2O and 5 µl of template DNA. Amplification was performed using an iCycler model thermocycler (Bio-rad, Hercules, CA) with the following program: initial denaturation (3.5 min at 95°C), 36 cycles of denaturation (30 sec at 95°C), annealing (50 sec at 60°C) and extension (40 sec at 72°C), and a final extension (5 min at 72°C). PCR products were detected on 1.5 percent agarose midigels by ethidium bromide staining and UV transillumination.

PCR products from mosquito bloodmeals and reference vertebrate tissue samples were digested using 4 restriction enzymes; *AciI, AluI, HaeIII* and *RsaI* (New England Biolabs, Beverly, MA). A chromosomal DNA preparation from the common grackle (*Quiscalus quiscala*) was used as a positive control. A 96 well PCR plate was divided into 24 blocks of 4 wells. Each well contained 9µl of enzyme master mix, containing 21.89 µg of control DNA, 0.97 µg of BSA, 0.24 µl of restriction enzyme and 0.97 µl of the respective NEBuffer (New England Biolabs, Beverly, MA). To each reaction, 1 µl of PCR product was added. Digestion was performed in an iCycler model thermocycler (Bio-rad) with the following program: 60 minutes at 37°C (enzyme

digest) and 20 minutes at 65°C (enzyme inactivation). Digestion products were then purified using the DyeEx 96 removal kit (Qiagen) according to the manufacturer's instructions.

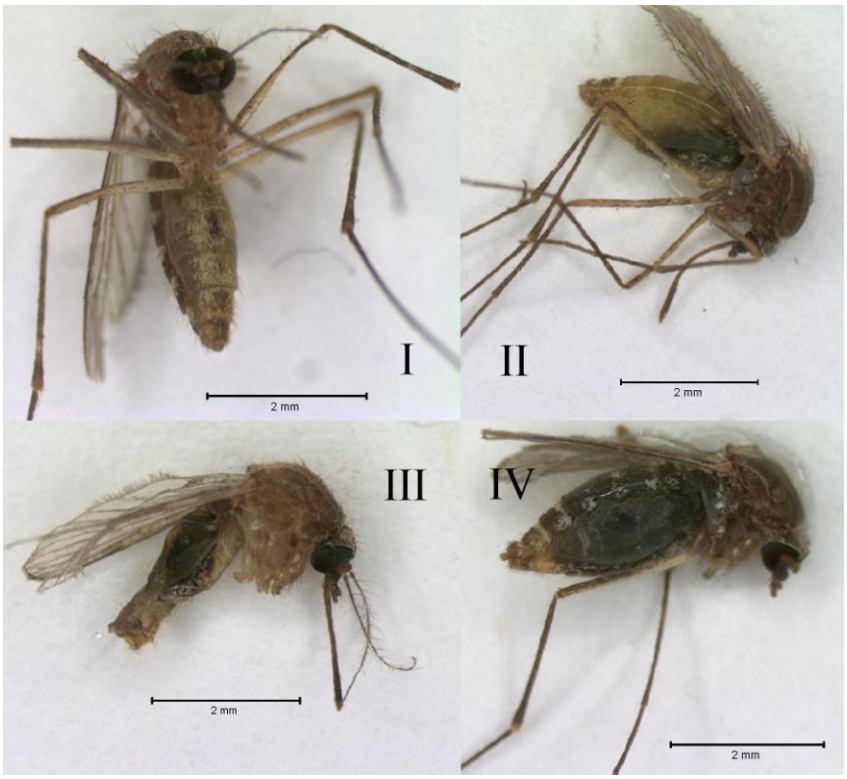

Figure 3. 1. Bloodmeal size grading scale; I = trace meal, no midgut distention, II = meal <1/3 of abdominal length, III = meal 1/3 – 2/3 of abdominal length, IV = fully engorged, abdomen greatly distended

Sizing of restriction fragments was performed by adding 14 µl of the GeneScan-500 size standards, in a 1:20 dilution of in Hi-Di formamide, and 1 µl of each digest product, to each well of a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA). The size standard mixture contained single-stranded DNA fragments of 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bases with a single ROX fluorophore. Samples were then denatured at 95°C for 2 minutes and immediately cooled to 4°C. Fragment lengths were measured in a model 3100 capillary DNA sequencing instrument using GeneMapper software (Applied Biosystems).

Direct sequencing was performed on samples that lacked a diagnostic TRF profile (no recognition sites for any of the four restriction enzymes), and samples with a TRF profile for the four enzymes that was shared by more than one host species. Sequencing also was used to confirm some of the species identifications provided by TRFLP. Identifications made initially by sequencing were confirmed by performing TRFLP analysis on a known sample of host tissue.

For sequencing, unlabelled BM1 and BM2 primers were used to amplify host *cytob* by PCR as described previously. Products were purified by polyethylene glycol (PEG) precipitation. Each 12 µl sequencing reaction contained 1 µl of purified PCR product, 3 µl of BigDye® Terminator version 3.1 ready reaction mix (Applied Biosystems), and 0.16 µM of unlabelled BM1 primer. Cycle sequencing was performed in an iCycler model thermocycler (Bio-rad, Hercules, CA) using the conditions described in the BigDye® Terminator v3.1 manual: initial denaturation at 96°C for 1 minute, followed by 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. Cycle sequence products were purified using the DyeEx 96 removal kit (Qiagen) according to the manufacturer's instructions. Sequence reactions were analyzed using a model 3100 capillary DNA sequencing instrument.

To determine host bloodmeal identity, the fragment length profiles for each digest was compared with a database (CYTBD) of fragment profiles for known host DNA samples. Field collected samples that failed to match a known fragment length profile were sequenced and compared to nucleic acid databases (GenBank) using the basic local alignment search tool (BLAST) service to find matches to host species.

The utilization of avian species as hosts, relative to their density in the Parish, was examined for *Cx. quinquefasciatus* using the Forage Ratio (FR) method (Hess *et al.* 1968). The FR was calculated by dividing the percentage of bloodmeals obtained by *Cx. quinquefaciatus*

from each host species by the percentage that the host species represents in the total avian community. Ninety-five percent confidence intervals for the forage ratios were computed as described by Lardeux *et al.* (2007). A FR value significantly greater than one is considered to indicate that the mosquito is exhibiting selective preference for that host species. A FR value significantly less than one is considered to indicate that the mosquito is exhibiting selective avoidance of the host.

Avian densities were estimated using data obtained from the North American Breeding Bird Survey (NABBS) for the Baton Rouge route in 2003 and 2004 (USGS 2007). These data are collected over a single day every June by an observer who makes point counts every 0.8 km along a 39.4 km route in the southern part of EBRP. For each point count, every bird seen or heard within a 0.8 km radius of the NABBS observer is recorded over a three minute period. Since the NABBS only collects data on wild bird species during the peak breeding season, domestic species (*Gallus gallus* and *Anser anser*) and winter resident species were excluded from the data sets used to calculate the numerator (feeding rate) and denominator (bird density) of the FR calculation.

In addition to calculating FR's using raw NABBS counts, biomass-adjusted FR's were obtained by multiplying the raw count value by the average body mass of an adult bird for each species, obtained from Dunning (1984). The proportion each species represented of the total estimated biomass of all wild, summer and permanent resident birds, was used as the denominator in the biomass-adjusted FR calculation.

Monthly proportions of *Cx. nigripalpus* and Cx. *quinquefasciatus* feeding on mammalian and avian hosts were compared by χ^2 analysis for trend using GraphPad Instat version 3.06 (GraphPad Software, San Diego, CA). Comparisons between the proportions of bloodmeals from

specific host species or groups, by year, were made using χ^2 analysis. The proportion of *Cx. quinquefasciatus* feeding on a human host in both years was compared using the Fisher's Exact Test.

3.3 Results

A total of 59 blood-fed *Cx. coronator*, 86 blood-fed *Cx. salinarius*, 200 blood-fed *Cx. nigripalpus*, and 1097 blood-fed *Cx. quinquefasciatus* were processed by T-RFLP, by sequencing, or by both T-RFLP and sequencing (Table 3.2). Among the four species, sufficient amplification of host DNA to produce an unambiguous TFR profile and/or sequence was achieved in approximately two-thirds to more than three-quarters of all bloodmeals processed. The influence of bloodmeal size on amplification success was significant by χ^2 analysis in *Cx. nigripalpus* (x^2=35.4, df=3, p≤0.001) and *Cx. quinquefasciatus*, (x^2=29.6, df=3, p≤0.001).

The proportion of bloodmeals identified as of avian or mammalian origin varied greatly among the mosquito species. The majority of identified *Cx. coronator*, *Cx. salinarius*, and *Cx. nigripalpus* bloodmeals were from a mammalian host (Table 3.3). However, only about 40 percent of identified *Cx. quinquefasciatus* bloodmeals were from a mammalian host, which was significantly lower by χ^2 analysis (p<0.0001) than the other three species. Host DNA from more than one vertebrate species was detected in a small proportion of blood-fed *Cx. salinarius*, *Cx. quinquefasciatus* and *Cx. nigripalpus*. None of the host DNA extracted from *Culex* bloodmeals was identified as originating from an animal belonging to a vertebrate class other than aves or mammalia.

The White-tailed Deer was the most frequently identified host of *Cx. coronator*, representing almost three-quarters of all identified bloodmeals (Table 3.4). Only two avian meals

were detected. The six host species, medium or large-sized mammals, were identified from the remaining *Cx. coronator* bloodmeals.

A total of 13 mammalian species, ranging in size from bovines and equines to small rodents, were identified from DNA extracted from blood-fed *Cx. salinarius* (Table 3.5). The White-tailed Deer and the Northern Raccoon were the most frequently identified hosts of *Cx. salinarius*, accounting for over 40 percent of all identified bloodmeals. Feline and canine hosts were the next most frequent hosts of *Cx. salinarius*. Blood meals from nine bird species were identified; the most common avian host was the domestic chicken. Bloodmeals of two *Cx. salinarius* contained DNA from multiple host species.

Table 3. 2. Blood-fed *Culex* females processed for bloodmeal identification.

	No. Females	% of Bloodmeals Successfully Amplified				
Species	Processed	Grade I	Grade II	Grade III	Grade IV	All BM
Cx. coronator	59	50.0	71.4	80.0	91.7	76.3
Cx. salinarius	86	64.7	76.5	95.7	91.7	81.4
Cx. nigripalpus	200	23.1	55.6	72.8	88.6	67.5
Cx. quinquefasciatus	1097	41.4	48.4	75.4	86.5	63.8

Table 3. 3. Identification of vertebrate host DNA from blood-fed *Culex* mosquitoes.

			% of Identified Samples			
			Single Host Species		Multiple Host Species[2]	
Species	No. Samples Amplified[1]	% Amplified Samples Identified	Aves	Mammalia	Both Classes	Single Class
Cx. coronator	45	82.2	5.4	94.6	0	0
Cx. salinarius	70	95.7	16.4	80.6	0	3.0
Cx. nigripalpus	136	82.4	33.9	64.3	1.8	0
Cx. quinquefasciatus	700	97.7	59.9	39.2	0.3	0.6

[1] Samples sufficiently amplified by PCR to produce a clear and unambiguous terminal restriction fragment profile, and / or sequence profile.
[2] DNA identified from more than one host in same blood-fed mosquito.

Almost two-thirds of all identified bloodmeals of *Cx. nigripalpus* were from medium and large mammals (Table 3.6). The most common mammalian hosts identified were the Northern

Raccoon, the White-tailed Deer, and the Virginia Opossum. These three hosts represented over half of all identified bloodmeals. More than 75 percent of avian meals, and one-quarter of all identified bloodmeals, were from passeriform birds. The most common avian hosts were the Northern Cardinal, the Common Grackle and the domestic chicken, which together represented almost one-fifth of the identified bloodmeals. The DNA from both mammalian and avian sources was identified in two *Cx. nigripalpus* bloodmeals.

Table 3. 4. Bloodmeal Hosts of *Culex coronator* collected from East Baton Rouge Parish, 2004.

Class	Species	Family	No. Blood meals	% of Identified Bloodmeals
Aves	Carolina Chickadee, *Poecile carolinensis*	Paridae	1	2.7
	Tufted Titmouse, *Baeolophus bicolor*	Paridae	1	2.7
	Total		**2**	**5.4**
Mammalia	White-tailed Deer, *Odocoileus virginianus*	Cervidae	27	73.0
	domestic horse, *Equus caballus*	Equidae	2	5.4
	Northern Raccoon, *Procyon lotor*	Procyonidae	2	5.4
	domestic cat, *Felis silvestris catus*	Felidae	1	2.7
	domestic dog, *Canis lupus familiaris*	Canidae	1	2.7
	River Otter, *Lontra canadensis*	Mustelidae	1	2.7
	Virginia Opossum, *Didelphis virginiana*	Didelphidae	1	2.7
	Total		**35**	**94.6**

DNA from 40 avian species of 9 orders was identified from blood-fed *Cx. quinquefasciatus* (Table 3.7). Over three-quarters of all identified avian bloodmeals were from passeriform species. Approximately 90 percent of avian bloodmeals were obtained from species that are permanent residents in East Baton Rouge Parish. Mammalian hosts represented approximately 40 percent of all identified bloodmeals of *Cx. quinquefasciatus*. The six most common mammalian hosts were the Northern Raccoon, domestic dog, human, the Virginia Opossum, domestic horse and domestic cat, which together represented over 90 percent of all mammalian

Table 3. 5. Blood Meal Hosts of *Culex salinarius* collected from sites in and adjacent to East Baton Rouge Parish, November, 2002, to November, 2004.

Class	Species	Family	No. Blood Meals	% of Identified Blood Meals	
				Class	All
Aves	domestic chicken, *Gallus gallus*	Phasianidae	3	25.0	4.5
	Cedar Waxwing, *Bombycilla cedrorum*	Bombycillidae	2	16.7	3.0
	Northern Cardinal, *Cardinalis cardinalis*	Fringillidae	2	16.7	3.0
	Barred Owl, *Strix varia*	Strigidae	1	8.3	1.5
	Field Sparrow, *Spizella pusilla*	Fringillidae	1	8.3	1.5
	Great Blue Heron, *Ardea herodias*	Ardeidae	1	8.3	1.5
	Northern Mockingbird, *Mimus polyglottos*	Sturnidae	1	8.3	1.5
	Wild Turkey, *Meleagris gallopavo*	Phasianidae	1	8.3	1.5
	Yellow-crowned Night-heron	Ardeidae	1	8.3	1.5
	Total[1]		**12**		**17.9**
Mammalia	White-tailed Deer, *Odocoileus virginianus*	Cervidae	17	30.9	25.4
	Northern Raccoon, *Procyon lotor*	Procyonidae	12	21.8	17.9
	domestic dog, *Canis lupus familiaris*	Canidae	8	14.5	11.9
	domestic cat, *Felis silvestris catus*	Felidae	6	10.9	9.0
	domestic cow, *Bos taurus*	Bovidae	4	7.3	6.0
	domestic horse, *Equus caballus*	Equidae	2	3.6	3.0
	domestic rabbit, *Oryctolagus cuniculus*	Leporidae	2	3.6	3.0
	Golden Mouse, *Ochrotomys nuttalli*	Muridae	1	1.8	1.5
	Hispid Cotton Rat, *Sigmodon hispidus*	Muridae	1	1.8	1.5
	Nutria, *Myocastor coypus*	Myocastoridae	1	1.8	1.5
	Roof Rat, *Rattus rattus*	Muridae	1	1.8	1.5
	Virginia Opossum, *Didelphis virginiana*	Didelphidae	1	1.8	1.5
	Total[2]		**55**		**82.1**

[1] Includes one mixed meal containing both Cedar Waxwing and Field Sparrow DNA.
[2] Includes one mixed meal containing both White-tailed Deer and domestic dog DNA.

Table 3. 6. Hosts of *Culex nigripalpus* collected from sites in and adjacent to East Baton Rouge Parish, November, 2002, to November, 2004.

Class	Species	Family	No. Blood Meals	% of Identified Blood Meals	
				Class	All
Aves	Northern Cardinal, *Cardinalis cardinalis*	Fringillidae	9	22.5	8.0
	Common Grackle, *Quiscalus quiscula*	Fringillidae	8	20.0	7.1
	domestic chicken, *Gallus gallus*	Phasianidae	6	15.0	5.4
	Blue Jay, *Cyanocitta cristata*	Corvidae	2	5.0	1.8
	Gray Catbird, *Dumetella carolinensis*	Sturnidae	2	5.0	1.8
	Summer Tanager, *Piranga rubra*	Fringillidae	2	5.0	1.8
	American Goldfinch, *Carduelis tristis*	Fringillidae	1	2.5	0.9
	Black Vulture, *Coragyps atratus*	Ciconiidae	1	2.5	0.9
	Brown Thrasher, *Toxostoma rufum*	Sturnidae	1	2.5	0.9
	Carolina Chickadee, *Poecile carolinensis*	Paridae	1	2.5	0.9
	European Starling, *Sturnus vulgaris*	Sturnidae	1	2.5	0.9
	Fish Crow, *Corvus ossifragus*	Corvidae	1	2.5	0.9
	Mandarin Duck, *Aix galericulata*	Anatidae	1	2.5	0.9
	Red-eyed Vireo, *Vireo olivaceus*	Vireonidae	1	2.5	0.9
	Red-winged Blackbird, *Agelaius phoeniceus*	Fringillidae	1	2.5	0.9
	White-eyed Vireo, *Vireo griseus*	Vireonidae	1	2.5	0.9
	Wild Turkey, *Meleagris gallopavo*	Phasianidae	1	2.5	0.9
	Total[1]		**40**		**35.7**
Mammalia	Northern Raccoon, *Procyon lotor*	Procyonidae	41	55.4	36.6
	White-tailed Deer, *Odocoileus virginianus*	Cervidae	13	17.6	11.6
	Virginia Opossum, *Didelphis virginiana*	Didelphidae	8	10.8	7.1
	Human, *Homo sapiens*	Hominidae	3	4.1	2.7
	domestic dog, *Canis lupus familiaris*	Canidae	2	2.7	1.8
	domestic cat, *Felis silvestris catus*	Felidae	2	2.7	1.8
	domestic cow, *Bos taurus*	Bovidae	2	2.7	1.8
	domestic horse, *Equus caballus*	Equidae	2	2.7	1.8
	nutria, *Myocastor coypus*	Myocastoridae	1	1.4	0.9
	Total[1]		**74**		**66.1**

[1] Includes 2 mixed meals; one containing both Common Grackle and White-tailed Deer DNA, one containing both Northern Cardinal and human DNA.

bloodmeals (Table 3.8). Host DNA from multiple species was detected in six blood-fed *Cx. quinquefasciatus*.

The proportion of bloodmeals obtained from domestic and wild mammalian hosts differed significantly among the four *Culex* species (χ^2=46.6, df=3, p<0.001; Table 3.9). The dominant mammalian hosts were large-sized, wild species for *Cx. coronator*, and medium-sized, wild species for *Cx. nigripalpus*. For both of these mosquito species, domestic hosts represented less than 15 percent of all mammalian meals. *Culex salinarius* fed about equally on three mammalian types: medium-sized, domestic mammals, medium-sized, wild mammals, and large-sized, wild mammals. Over half of all *Cx. quinquefasciatus* mammalian bloodmeals were from domestic species; most of the remaining mammalian bloodmeals were from medium-sized, wild species.

A seasonal shift in the host feeding behavior of *Cx. nigripalpus* was not observed (Fig. 3.2). In 2003, no significant differences were detected, by χ^2 analysis for linear trend, in the proportion of identified *Cx. nigripalpus* bloodmeals obtained from mammalian hosts, by month, from August to November (χ^2=0.02, df=1, p=0.89). In 2004, the proportions of identified *Cx. nigripalpus* bloodmeals obtained from mammalian hosts in October and November were not significantly different by χ^2 analysis (χ^2=0, df=1, p=1.0).

From May though October, the proportion of hosts identified from bloodfed *Cx. quinquefasciatus* that were mammalian ranged from 24 to 48.3 percent in 2003, and from 17.4 to 48.5 percent in 2004 (Fig. 3.3). There was no significant difference, detected by χ^2 analysis for linear trend, in the monthly proportion of *Cx. quinquefasciatus* feeding on mammals, in 2003 (χ^2=0.07, df=1, p=0.79), or 2004 (χ^2=3.33, df=1, p=0.07). Similarly, no significant shift in the proportion of *Cx. quinquefasciatus* feeding on humans was detected by χ^2 analysis for linear

Table 3. 7. Avian Hosts of *Culex quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

			% of Blood Meals	
Species	Family	No.	Avian	All
Northern Cardinal, *Cardinalis cardinalis*	Fringillidae	102	24.8	14.9
Northern Mockingbird, *Mimus polyglottos*	Sturnidae	62	15.0	9.1
Common Grackle, *Quiscalus quiscula*	Fringillidae	43	10.4	6.3
Mourning Dove, *Zenaida macroura*	Columbidae	33	8.0	4.8
domestic chicken, *Gallus gallus*	Phasianidae	28	6.8	4.1
House Sparrow, *Passer domesticus*	Passeridae	22	5.3	3.2
Brown Thrasher, *Toxostoma rufum*	Sturnidae	16	3.9	2.3
Blue Jay, *Cyanocitta cristata*	Corvidae	13	3.2	1.9
Tufted Titmouse, *Baeolophus bicolor*	Paridae	9	2.2	1.3
Fish Crow, *Corvus ossifragus*	Corvidae	8	1.9	1.2
Carolina Chickadee, *Poecile carolinensis*	Paridae	7	1.7	1.0
Yellow-billed Cuckoo, *Coccyzus americanus*	Cuculidae	7	1.7	1.0
Barred Owl, *Strix varia*	Strigidae	5	1.2	0.7
Gray Catbird, *Dumetella carolinensis*	Sturnidae	5	1.2	0.7
Purple Martin, *Progne subis*	Hirundinidae	5	1.2	0.7
Green Heron, *Butorides virescens*	Ardeidae	4	1.0	0.6
Mallard Duck, *Anas platyrhynchos*	Anatidae	4	1.0	0.6
Summer Tanager, *Piranga rubra*	Fringillidae	4	1.0	0.6
Eastern Bluebird, *Sialia sialis*	Muscicapidae	3	0.7	0.4
European Starling, *Sturnus vulgaris*	Sturnidae	3	0.7	0.4
Red-eyed Vireo, *Vireo olivaceus*	Vireonidae	3	0.7	0.4
Red Shouldered Hawk, *Buteo lineatus*	Accipitridae	3	0.7	0.4
Blue-Gray Gnatcatcher, *Polioptila caerulea*	Certhiidae	2	0.5	0.3
Great Blue Heron, *Ardea herodias*	Ardeidae	2	0.5	0.3
White-eyed Vireo, *Vireo griseus*	Vireonidae	2	0.5	0.3
Wood Thrush, *Hylocichla mustelina*	Muscicapidae	2	0.5	0.3
American Redstart, *Setophaga ruticilla*	Fringillidae	1	0.2	0.1
Brewer's Blackbird, *Euphagus cyanocephalus*	Fringillidae	1	0.2	0.1
Carolina Wren, *Thryothorus ludovicianus*	Certhiidae	1	0.2	0.1
Downey Woodpecker, *Picoides pubescens*	Picidae	1	0.2	0.1
Eastern Meadowlark, *Sturnella magna*	Fringillidae	1	0.2	0.1
Grasshopper Sparrow, *Ammodramus savannarum*	Fringillidae	1	0.2	0.1
Great Horned Owl, *Bubo virginianus*	Strigidae	1	0.2	0.1
Greylag Goose, *Anser anser*	Anatidae	1	0.2	0.1
Little Blue Heron, *Egretta caerulea*	Ardeidae	1	0.2	0.1
Red-Bellied Woodpecker, *Melanerpes carolinus*	Picidae	1	0.2	0.1
Red-winged Blackbird, *Agelaius phoeniceus*	Fringillidae	1	0.2	0.1
Swamp Sparrow, *Melospiza georgiana*	Fringillidae	1	0.2	0.1
Wild Turkey, *Meleagris gallopavo*	Phasianidae	1	0.2	0.1
Yellow-breasted Chat, *Icteria virens*	Fringillidae	1	0.2	0.1
Total[1]		**412**		**60.2**

[1] Includes 2 mixed meals; one containing both Northern Mockingbird and human DNA, one containing both Carolina Chickadee and Northern Raccoon DNA.

trend, in 2003 (χ^2=2.01, df=1, p=0.16), or 2004 (χ^2=1.62, df=1, p=0.20). In both years, the month with the highest proportion of *Cx. quinquefasciatus* that obtained blood from a mammalian host was August.

Table 3. 8. Mammalian Hosts of *Culex quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

Species	Family	No. Blood Meals	% of Identified Blood Meals	
			Mammalian	All
Northern Raccoon, *Procyon lotor*	Procyonidae	79	28.8	11.5
domestic dog, *Canis lupus familiaris*	Canidae	57	20.8	8.3
Human, *Homo sapiens*	Hominidae	48	17.5	7.0
Virginia Opossum, *Didelphis virginiana*	Didelphidae	41	15.0	6.0
domestic horse, *Equus caballus*	Equidae	19	6.9	2.8
domestic cat, *Felis silvestris catus*	Felidae	11	4.0	1.6
domestic cow, *Bos taurus*	Bovidae	5	1.8	0.7
Nine-banded Armadillo, *Dasypus novemcinctus*	Dasypodidae	5	1.8	0.7
White-tailed Deer, *Odocoileus virginianus*	Cervidae	4	1.5	0.6
domestic pig, *Sus scrofa domestica*	Suidae	3	1.1	0.4
Hispid Cotton Rat, *Sigmodon hispidus*	Muridae	2	0.7	0.3
Fox Squirrel, *Sciurus niger*	Sciuridae	1	0.4	0.1
River Otter, *Lontra canadensis*	Mustelidae	1	0.4	0.1
Roof Rat, *Rattus rattus*	Muridae	1	0.4	0.1
Total[1]		**274**		**40.1**

[1] Includes 6 mixed meals; one containing both Northern Mockingbird and human DNA, one containing both Carolina Chickadee and Northern Raccoon DNA, one containing domestic dog and Virginia Opossum DNA, one containing domestic dog and unidentified DNA, two containing domestic dog and Northern Raccoon DNA.

The seasonal utilization of specific host species by *Cx. quinquefasciatus* was examined. The two most common avian hosts of *Cx. quinquefasciatus*, the Northern Cardinal and the Northern Mockingbird, together accounted for approximately 16 to 31 % of all identified bloodmeals from *Cx. quinquefasciatus* collected each month, from May through November (Fig. 3.4). Approximately a third of all identified bloodmeals from *Cx. quinquefasciatus* collected in March and December contained Northern Cardinal DNA. Blue Jays and Common Grackles were

most important as hosts of *Cx. quinquefasciatus* in the winter and spring months (Fig. 3.5). The proportion of bloodmeals represented by Mourning Doves was greatest in the spring.

For *Cx. quinquefasciatus*, the proportion of identified bloodmeals that were human-derived increased from 2.7 percent in 2003, to 9.4 percent in 2004. This difference was significant by Fisher's Exact Test (p=0.001). A greater number of blood-fed *Cx. quinquefasciatus* were collected and identified in 2004 (n=415) than in 2003 (n=263). The proportion of identified *Cx. quinquefasciatus* bloodmeals collected at each site, as a proportion of the total number of identified *Cx. quinquefasciatus* bloodmeals, was compared between the two years to determine if differences in sampling success at each site, between the two years, could have skewed the human feeding rate (Table 3.10). The proportions of the total identified *Cx. quinquefasciatus* bloodmeals that were collected from the Ednie Lane and Greenwell Springs Road sites were significantly less in 2004 than it was in 2003. The proportions of the total identified *Cx. quinquefasciatus* bloodmeals that were collected from the Farr Park and Pecue Lane sites both increased significantly, from 2003 to 2004. Two additional sites, Clinton and Slaughter, were not sampled in 2004. When these six sites were excluded from the analysis, the increase in the proportion of identified *Cx. quinquefasciatus* bloodmeals obtained from a human host, from 3.3 percent in 2003 to 9.9 percent in 2004, was still significant (p= 0.019). In both years, there were no significant differences in the human feeding rate detected among the five habitat groups, or between sites designated as residential and non-residential.

A total of 375 *Cx. quinquefasciatus* bloodmeals, representing approximately 98 percent of the *Cx. quinquefasciatus* bloodmeals identified from wild birds and 91 percent of all *Cx. quinquefasciatus* avian bloodmeals, were used to calculate FR values. Permanent and summer residents represented more than 99 percent of all birds in the NABBS data sets for 2003 and

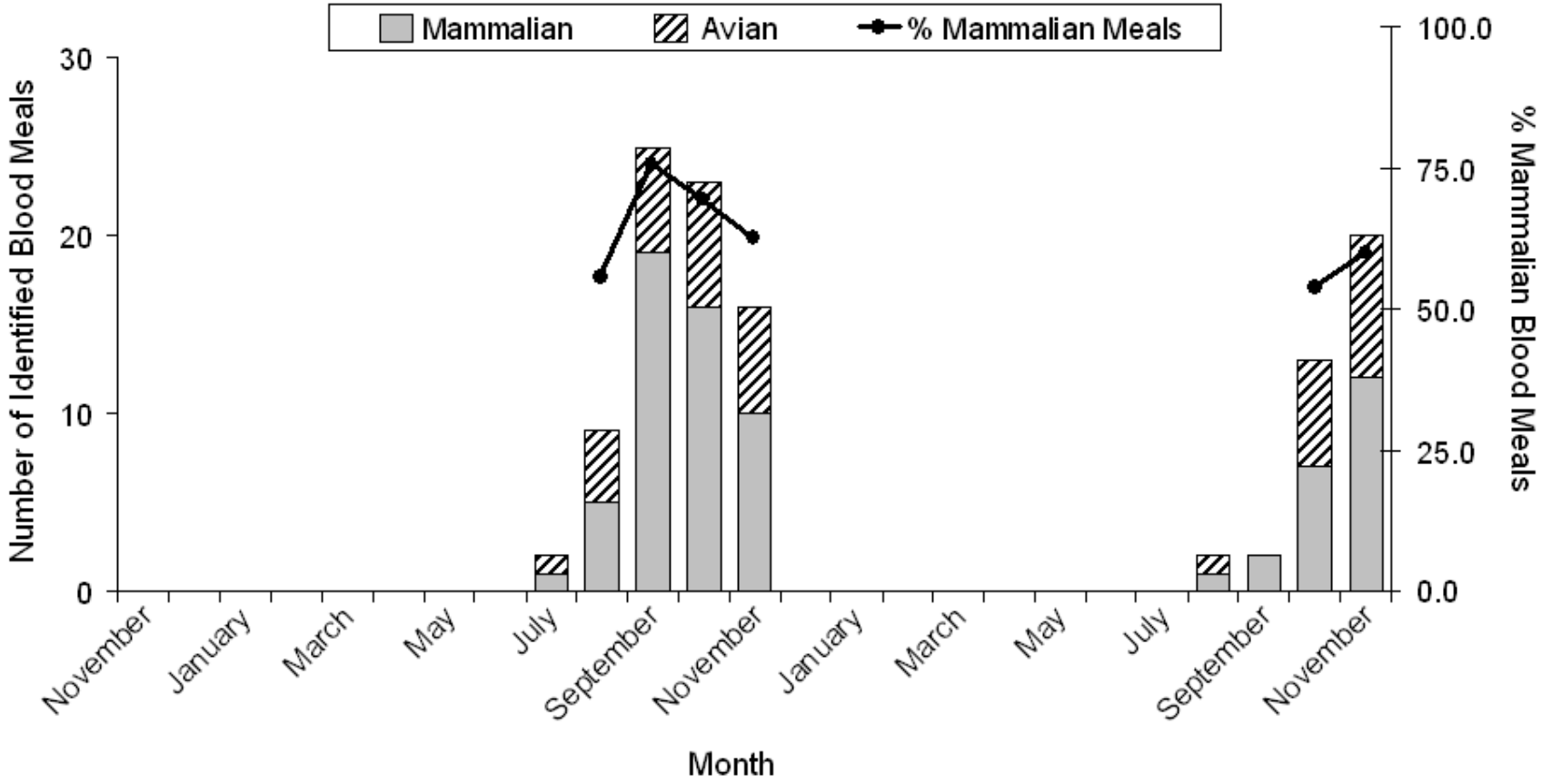

Figure 3. 2. Seasonal host utilization by *Cx. nigripalpus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

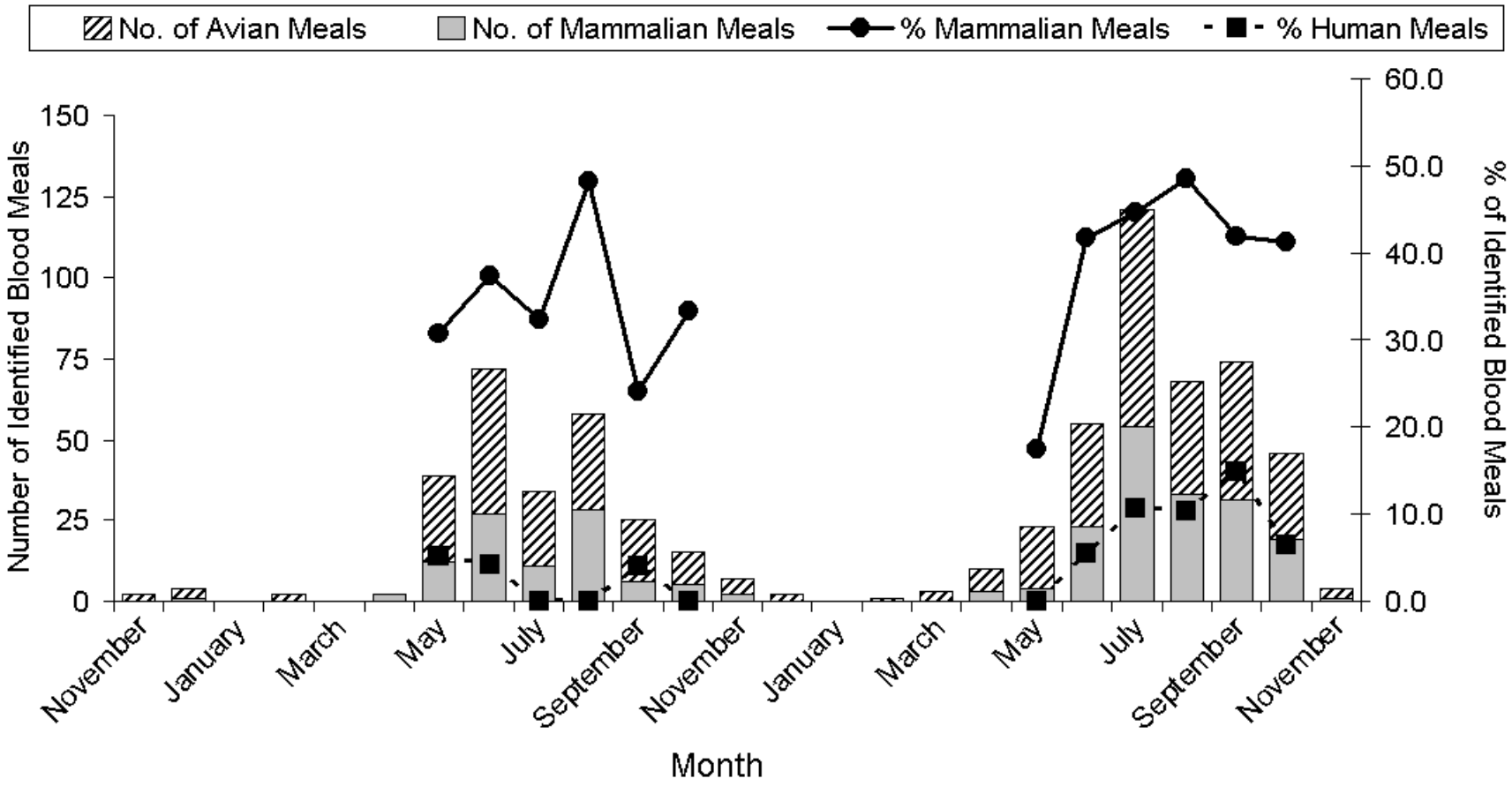

Figure 3. 3. Seasonal host utilization by *Cx. quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

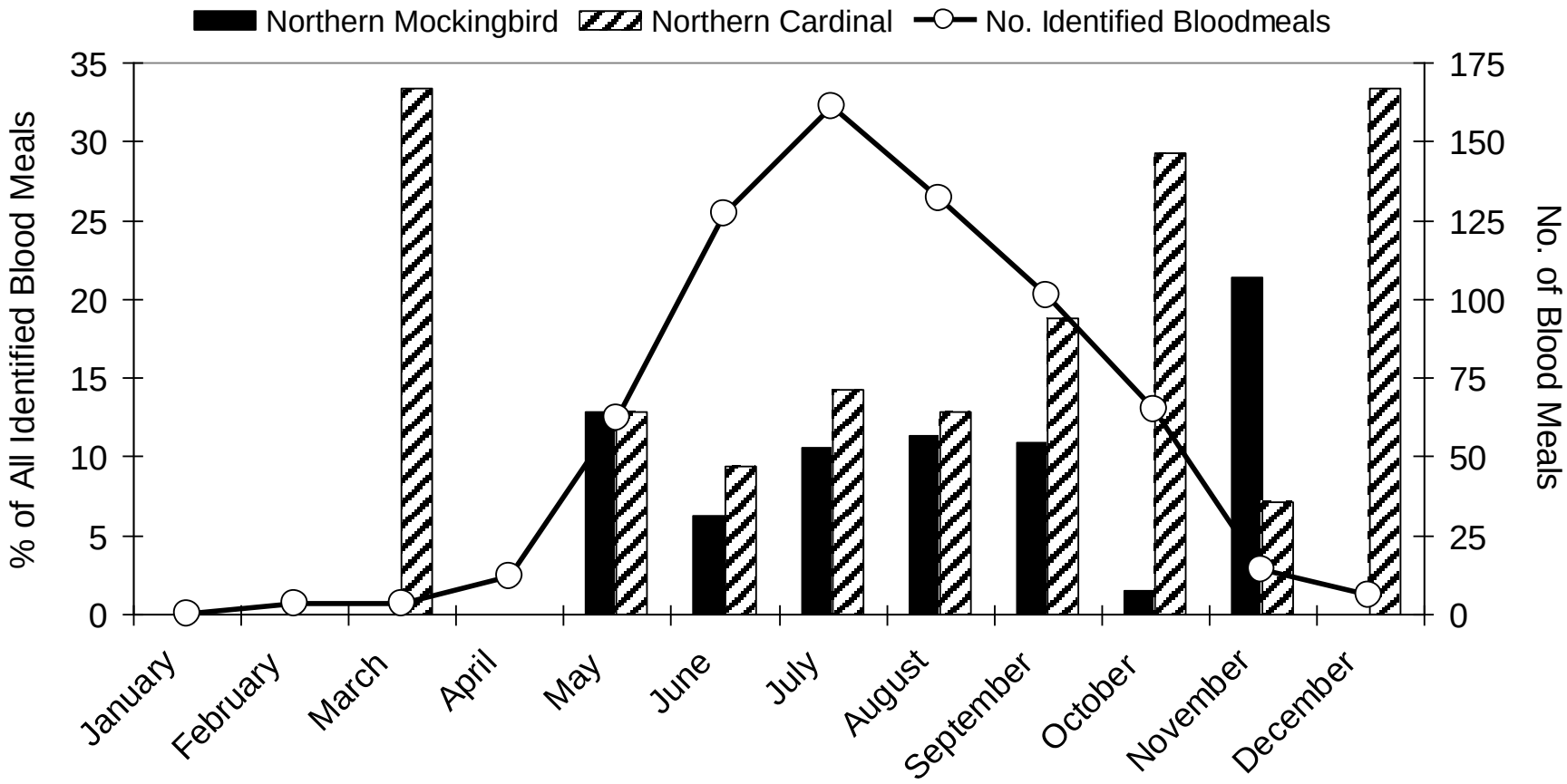

Figure 3. 4. Proportion of total identified bloodmeals obtained from Northern Cardinal and Northern Mockingbird per month, by *Cx. quinquefasciatus*, from November, 2002, to November, 2004.

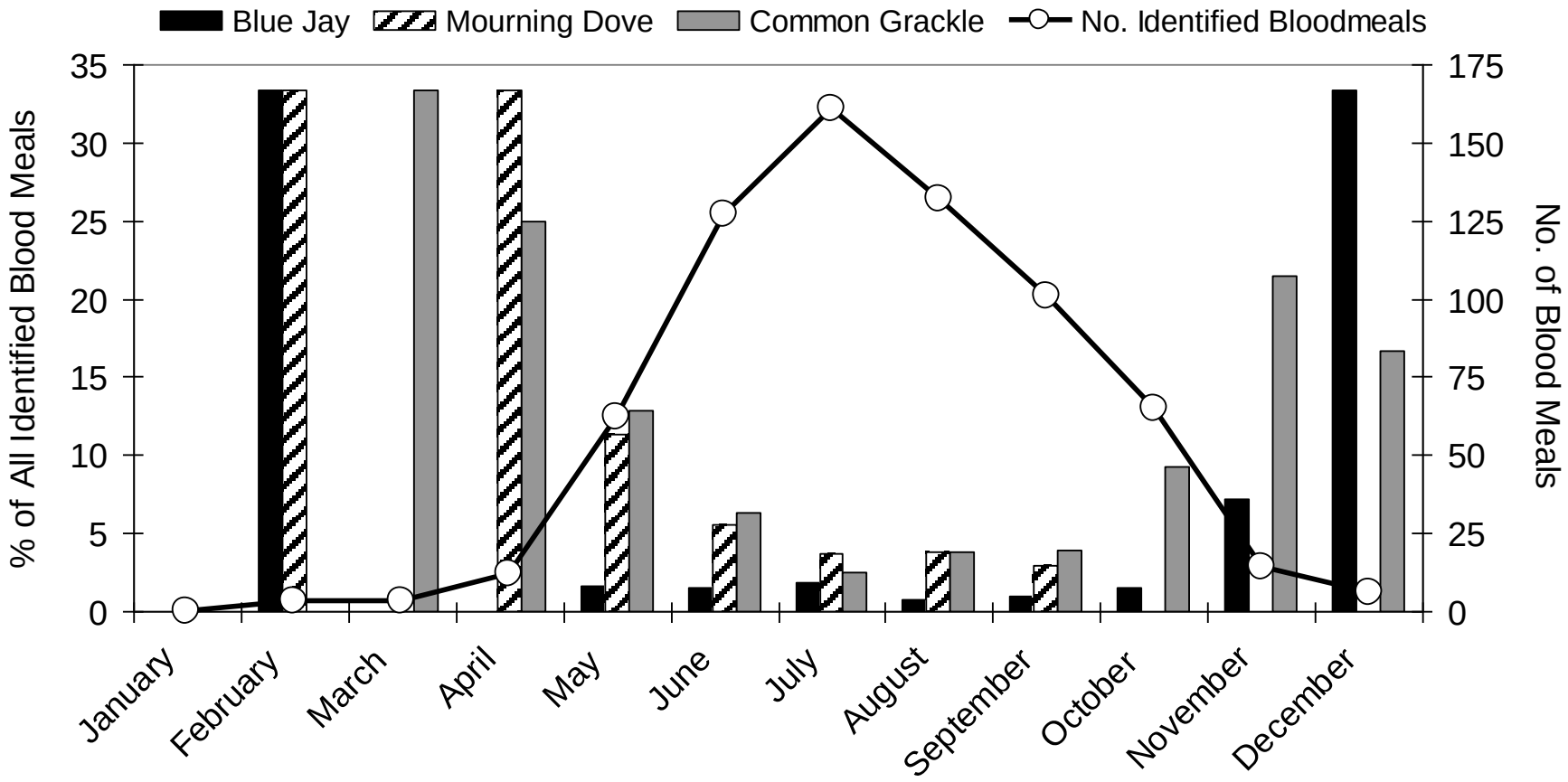

Figure 3. 5. Proportion of total identified bloodmeals obtained from Blue Jays, Mourning Doves and Common Grackles per month, by *Cx. quinquefasciatus*, from November, 2002, to November, 2004.

2004. Relative host preference was examined in avian species detected in at least four *Cx. quinquefasciatus* bloodmeals (Table 3.11). Forage Ratios also were calculated for common, wild, permanent and summer resident avian species (ie. representing at least one percent of total NABBS counts), but infrequently or never detected in *Cx. quinquefasciatus* bloodmeals (Table 3.12).

Table 3. 9. Size Distribution[1] of Mammalian Hosts of *Culex* species collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

	Proportion of Mammalian Blood Meals						
	Domestic Species			Wild Species			
Species	medium	large	**total**	small	medium	large	**total**
Cx. coronator	5.8	5.7	**11.5**	0	11.5	77.1	**88.6**
Cx. nigripalpus	5.4	9.5	**14.9**	0	67.6	17.6	**85.1**
Cx. salinarius[2]	29.1	10.9	**40.0**	5.5	25.5	30.9	**61.9**
Cx. quinquefasciatus[3]	24.8	27.4	**52.2**	1.5	46.0	1.5	**49.0**

[1] small hosts (avg. wt. < 1.5 kg) = all rodent species, except nutria; medium hosts (1.5 kg < avg. wt. < 40 kg) = domestic dog, domestic cat, domestic rabbit, Northern Raccoon, Virginia Opossum, Nutria, Nine-banded Armadillo, and River Otter; large hosts (avg. wt. > 40 kg) = domestic horse, domestic cow, domestic pig, human, and White-tailed Deer.
[2] total >100 percent; includes a mixed meal containing domestic dog and White-tailed Deer blood.
[3] total >100 percent; includes a mixed meal containing both domestic dog & Virginia Opossum DNA, and two mixed meals containing both domestic dog and Northern Raccoon DNA.

The non-adjusted FR values calculated for the Northern Cardinal, the Northern Mockingbird, the Common Grackle, the Brown Thrasher, the Tufted Titmouse, and the Yellow-billed Cuckoo, were significantly greater than one, indicating that these species were fed on at a greater rate than expected based on their abundance (Table 3.11). The non-adjusted FR values calculated for the House Sparrow, the Purple Martin, the European Starling, the Carolina Wren and the Red-bellied Woodpecker, were significantly less than one, indicating that these species were fed on at a lower rate than expected based on their abundance (Table 3.11, Table 3.12). Seven avian species fed on by *Cx. quinquefasciatus*, representing a total of 15 bloodmeals, were

Table 3. 10. Comparison of engorged *Cx. quinquefasciatus* sampling success and human blood meal identification by site.

Habitat Type	Site	No. of Identified Blood Meals 2003	No. of Identified Blood Meals 2004	% of Total Identified Blood Meals Δ[1]	% of Total Identified Blood Meals p-value[2]	No. of Human Blood Meals (% of total identified meals collected from site) 2003	No. of Human Blood Meals (% of total identified meals collected from site) 2004
Residential & Wooded & Swamp	Greenwell Springs Road	37	35	**-5.6**	**0.043**	1 (2.7)	4 (11.4)
	Lee High School	7	22	2.6	n.s.	0	2 (9.1)
Residential & Wooded	Blackwater Road	21	28	-1.2	n.s.	1 (4.8)	5 (17.9)
	Clinton[3]	0	-	-		0	Not sampled
	Denham Road	18	39	2.6	n.s.	0	3 (7.7)
	Ednie Lane	20	6	**-6.2**	**0.0001**	0	0
	Highland Road	10	12	-0.9	n.s.	0	1 (8.3)
	Hoo Shoo Too Road	5	5	-0.7	n.s.	0	0
	Oneal Lane	10	23	1.7	n.s.	0	1 (4.4)
	Pecue Lane	11	46	**6.9**	**0.004**	0	3 (6.5)
	Strain Road	5	11	0.7	n.s.	0	2 (18.20
Residential	Emmet Bourgeois Road	30	62	3.5	n.s.	2 (6.7)	5 (8.1)
	Slaughter[3]	26	-	-		1	Not sampled
Wooded	City Park	11	13	-1.0	n.s.	0	4 (30.8)
	Greenwood Park	3	0	-1.1	n.s.	1 (33.3)	0
	St. Gabriel Research Stn.	0	1	0.2	n.s.	0	0
Horse Stable	Farr Park	19	65	**8.4**	**0.0039**	0	6 (9.2)
	Lazy B Stables	30	47	-0.1	n.s.	1 (3.3)	3 (6.4)
	Total	**263**	**415**			**7 (2.7)**	**39 (9.2)**

[1] Change in % of total identified blood meals = ((no. identified blood meals from site *i* in 2004 / total no. identified blood meals in 2004) – (no. identified blood meals from site *i* in 2003 / total no. identified blood meals in 2003)) * 100

[2] Tested by Fisher's Exact Test; n.s. = not significant (p-value>0.05)

[3] Site not sampled in 2004.

not represented in the NABBS counts in 2003 or 2004. Host DNA from a total of 30 permanent and summer resident species, collectively representing approximately 26 % of all permanent and summer residents counts reported to the NABBS in 2003 and 2004, was not detected in any blood-fed *Cx. quinquefasciatus*. When adjusted for body mass, the FR's of the House Sparrow and the Carolina Chickadee were also greater than one, but the FR's of the Fish Crow, the European Starling and the Carolina Wren were significantly less than one.

Table 3. 11. Forage ratios for permanent and summer resident, wild avian species most frequently detected in bloodfed *Cx. quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

Species	% of Blood Meals	% of Avian Population (Rank)[1]	Forage Ratio (95% C.I.) Non-adjusted	Biomass-adjusted
Northern Cardinal	27.2	9.4 (3)	2.9 (2.5 – 3.3)	7.8 (6.7 – 8.9)
Northern Mockingbird	16.5	9.3 (4)	1.8 (1.4 – 2.1)	4.4 (3.5 – 5.2)
Common Grackle	11.5	7.3 (5)	1.6 (1.2 – 2.0)	1.7 (1.3 – 2.1)
Morning Dove	8.8	6.2 (6)	1.4 (1.0 – 1.8)	1.4 (1.0 – 1.8)
House Sparrow	5.9	12.1 (1)	0.5 (0.3 – 0.6)	2.1 (1.4 – 2.8)
Brown Thrasher	4.3	1.7 (15)	2.5 (1.5 – 3.5)	4.4 (2.6 – 6.2)
Blue Jay	3.5	4.0 (9)	0.9 (0.5 – 1.3)	1.2 (0.7 – 1.8)
Tufted Titmouse	2.4	1.2 (17)	2.0 (0.9 – 3.1)	11.2 (5.1 – 17.2)
Fish Crow	2.1	3.2 (12)	0.7 (0.3 – 1.1)	0.3 (0.1 – 0.5)
Carolina Chickadee	1.9	1.9 (14)	1.0 (0.4 – 1.6)	11.2 (4.3 – 18.1)
Yellow Billed Cuckoo	1.9	0.1 (28)	17.0 (6.5 – 27.4)	31.6 (12.1 – 51.1)
Barred Owl	1.3	0		
Purple Martin	1.3	4.3 (8)	0.3 (0.1 – 0.5)	0.7 (0.2 – 1.1)
Green Heron	1.1	0.5 (22)	2.2 (0.4 – 3.9)	1.2 (0.2 – 2.3)
Mallard	1.1	0		
Summer Tanager	1.1	0		

[1] Only wild, summer and permanent resident species used to calculate percentages and ranks. Some rank values contain multiple species.

Indices for the avian feeding preference of *Cx. quinquefasciatus* also were calculated by size class (Table 3.13). Non-adjusted FR values were significantly less than one for the smallest (species of average mass < 30 g) and largest (species of average mass ≥ 200 g) avian host size classes. The FR of the smallest size class was greater than one when adjusted for biomass. The

size class representing avian species with an average mass between 50 and 200 g had a FR of approximately one, indicating that they were fed on proportional to their abundance. The non-adjusted and biomass adjusted FR values for the size class containing avian species with an average mass between 30 and 50 g, was significantly greater than one, indicating that they were fed on at a rate greater than expected, based on their relative abundance and biomass.

Table 3. 12. Forage ratios for common[1], permanent and summer resident, wild avian species infrequently, or never, detected in bloodfed *Cx. quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

Species	% of Blood Meals	% of Avian Population (Rank)[2]	Forage Ratio (95% C.I.) Non-adjusted	Biomass-adjusted
Chimney Swift, *Chaetura pelagica*	0	10.5 (2)		
Carolina Wren	0.3	4.8 (7)	<0.1 (0.0 – 0.1)	0.3 (0.0 – 0.8)
European Starling	0.8	3.8 (10)	0.2 (0.0 – 0.4)	0.3 (<0.1 – 0.6)
Mississippi Kite, *Ictinia mississippiensis*	0	3.5 (11)		
Red-winged Blackbird, *Agelaius phoeniceus*	0	2.7 (13)		
Brown-headed Cowbird, *Molothrus ater*	0	1.3 (16)		
Red-bellied Woodpecker	0.3	1.2 (17)	0.2 (0.0 – 0.6)	0.4 (0.0 – 1.1)
Black Vulture, *Coragyps atratus*	0	1.1 (18)		
Wood Duck, *Aix sponsa*	0	1.1 (18)		

[1] Species representing at least one percent of the total Breeding Bird Survey counts of permanent and summer resident species for the Baton Rouge route, 2003 and 2004.

3.4 Discussion

The range of *Cx. coronator* in the United States was until recently described as limited to the southern parts of Texas and New Mexico, and southeastern Arizona (Darsie and Ward 2005). The first confirmed collection of specimens of this species from Louisiana was in 2004 (Debboun *et al.* 2005), however *Cx. coronator* females were collected in the current study from EBR, East Feliciana and Iberville Parishes from September to November, 2003 (A. Mackay,

pers. obs.). This species also was collected for the first time from locations in Mississippi in 2004, and Florida in 2005 (Goddard *et al.* 2006, Smith *et al.* 2006). The public health importance of *Cx. coronator* is unclear. Although WNV has been detected in *Cx. coronator* collected from EBR Parish (Table 2.4), the vector competence of this species for WNV has not been evaluated. In the Caribbean, and Central and South America, SLEV and VEEV have also been detected in *Cx. coronator* (Aitken *et al.* 1964, Scherer *et al.* 1971), although *Cx. coronator* from Peru have been shown to be poor vectors of VEEV (Turell et al. 2000a).

Table 3. 13. Avian host utilization of by size class of *Cx. quinquefasciatus* collected from sites in and adjacent to East Baton Rouge Parish, from November, 2002, to November, 2004.

Species Average Mass (g)[1]	No. Host Species[2]	% of Blood Meals	Avian Abundance		Avian Biomass	
			% of Total	Forage Ratio (95% C.I.)	% of Total	Forage Ratio
<30	19	14.4	33.0	0.44 (0.39 – 0.48)	6.5	2.22
30 to <50	9	45.1	21.3	2.11 (2.02 – 2.20)	8.2	5.63
50 to <200	13	33.3	32.6	1.02 (0.97 – 1.08)	24.1	1.39
>200	23	7.2	13.0	0.55 (0.48 – 0.63)	61.3	0.12

[1] Average weight of individual bird.
[2] Total number of wild, summer or permanent resident avian host species in size class identified in blood fed *Cx. quinquefasciatus* and NABBS counts.

In the current study, almost three-quarters of the identified bloodmeals from *Cx. coronator* were from White-tailed Deer, suggesting a feeding preference of this mosquito for large mammals. This pattern is consistent with previous observations on the feeding preference of *Cx. coronator* in the southwestern United States, and Central and South America. Large numbers of host-seeking females have been collected from equine hosts in Mexico, Texas and New Mexico (Jones *et al.* 1977, Reyes-Villanueva *et al.* 2006), and human hosts in Belize, Brazil and Mexico (Bertram 1971, Roberts *et al.* 1981, Reyes-Villanueva *et al.* 2006). Small numbers

of *Cx. coronator* have also been collected from rodent baited traps in western Texas (Easton *et al.* 1968). A sample of seven blood-fed *Cx. coronator* females collected from the southeastern region of the United States (presumably Texas) were examined by precipitin test, all reacted with rabbit antisera (Suyemoto *et al.* 1973). The host range described by our data and previous studies suggests that *Cx. coronator* is unlikely to contribute significantly to the enzootic amplification cycle of WNV, but is a potential bridge vector of WNV to equines, and possibly humans.

In host selection experiments, *Cx. salinarius* does not exhibit strong host specificity, readily feeding on both mammalian and avian hosts (Hayes 1961, Murphey *et al.* 1967). In the current study, the majority of identified *Cx. salinarius* bloodmeals were from mammalian hosts. However, a very wide diversity of host species was identified, suggesting an opportunistic feeding strategy. A significant proportion of bloodmeals were derived from domestic mammalian hosts, and approximately nine percent of the identified bloodmeals were from passeriform birds. This is consistent with previous host range studies reporting that this species typically obtains most of its blood meals from mammalian hosts. Suyemoto *et al.* (1973) reported that greater than 95 percent of blood-fed *Cx. salinarius* collected from Texas, Oklahoma, Arkansas and Louisiana had previously fed on a wide range of mammalian hosts. In salt marsh habitats in Louisiana, the most frequently utilized hosts were cattle (77%), rabbit (8%) and horse (6%), while only 3 percent of *Cx. salinarius* females were found to have fed on an avian host (Schaefer and Steelman 1969); the proportion of each host utilized was representative of the density of each host species in the study area. In North Carolina, approximately 71 percent of bloodmeals from *Cx. salinarius* were from a canine host, approximately 15 percent were from deer, and the remaining 14 percent were identified as avian (Irby and Apperson 1988). In New Jersey, 71.9, 24.6 and 3.5 percent of blood-fed *Cx. salinarius* collected from various locations were identified

as feeding on mammalian, avian and amphibian hosts, respectively; the most common hosts were deer, raccoon and human (Apperson *et al.* 2004). In Florida, Edman (1974) reported that the proportion of *Cx. salinarius* feeding on mammals varied greatly among locations, from 42 percent of blood-fed females collected from sites in Vero Beach to 93 percent of blood-fed females collected from sites in Tampa.

Although the larval habitats for *Cx. salinarius* are predominantly brackish, this species can be abundant East Baton Rouge Parish (Chapman and Johnson 1986). West Nile virus has been isolated from several pools containing female *Cx. salinarius* collected from the parish (Table 2.2). This species is considered a highly competent vector of WNV (Sardelis *et al.* 2001), and the results of the current study suggest that it may play a role as a bridge vector of WNV in EBRP.

This was the first study to examine the host range of *Cx. nigripalpus* in Louisiana, and the data suggest that *Cx. nigripalpus* has an opportunistic feeding strategy. Approximately two-thirds of the bloodmeals identified from blood-fed females were from mammalian hosts common to East Baton Rouge Parish. The remaining hosts identified in the current study included a diverse range of avian species, primarily passeriformes. This is consistent with host range data collected on *Cx. nigripalpus* populations in wetland and agricultural habitats at several sites in Florida (Edman 1974). Depending upon the local vertebrate faunal composition, the proportion of females feeding on mammalian hosts ranged by site from approximately 50 to over 90 percent, which suggests a highly opportunistic bloodfeeding strategy. The most common hosts identified from *Cx. nigripalpus* bloodmeals were representative of the most abundant vertebrate species at each study site (Edman 1974). Unlike the current study, wild mesopredators collectively represented only a small proportion of mammalian bloodmeals in Florida. Similarly

in an urban area in Brazil, host bloodmeals from 45.3 percent of blood-fed *Cx. nigripalpus* females reacted with anti-chicken antibodies, indicating an avian host (Gomes *et al.* 2003). The remaining bloodmeals reacted with anti-bovine (21.9%), anti-human (18.5%), anti-canine (10.8%), anti-equine (1.7%), anti-rat (1.7%), anti-pig (0.3%), and anti-feline (0.2%) antibodies. However a *Cx. nigripalpus* population studied in Panama has been reported to be primarily ornithophagic (Christensen *et al.* 1996); almost 90 percent of the bloodfed females tested had fed on a bird.

In Florida, *Cx. nigripalpus* is considered to be one of the most important epizootic vectors of WNV (Rutledge *et al.* 2003, Godsey *et al.* 2005a), and the primary enzootic and epidemic vector of SLEV in central and southern part of the state (Dow *et al.* 1964, Shroyer 1991). Previous studies in rural habitats in Florida have reported a very low rate of feeding on humans (Provost 1969), and very few host seeking *Cx. nigripalpus* females are observed in human landing collections, suggesting that humans are not a preferred host for this mosquito species (Bidlingmayer 1967). In the current study, a relatively low rate of anthropophagy was observed. In both 2003 and 2004, host-seeking females were not collected prior to July in EBRP, and not abundant until September (Chapter 2, data not shown). However, *Cx. nigripalpus* could still serve as a late season epidemic vector of WNV, as the onset of human WNV infection can extend into the late autumn in Louisiana (Balsamo *et al.* 2003). The timing of the seasonal activity of *Cx. nigripalpus*, and its frequent use of mammals as hosts, may limit the importance of this mosquito species to the enzootic amplification of WNV in southern Louisiana.

In the current study, over 60 percent of identified *Cx. quinquefasciatus* bloodmeals contained avian DNA, mostly from passeriform species, which provides further evidence that *Cx. quinquefasciatus* is the primary enzootic vector of WNV in EBRP (Gleiser *et al.* 2007). The

frequency of avian feeding may have been influenced by the types of habitats where the majority of *Cx. quinquefasciatus* were collected in our study. More than half of the sites sampled were located within, or close to, hardwood forests. In a previous host range study of *Cx. quinquefasciatus* in East Baton Rouge Parish, avian blood was detected in less than 20 % of females collected directly adjacent to human residences, but in over 45 % of females collected from hardwood forests (Niebylski and Meek 1992). In Florida, only 33 % of blood-fed *Cx. quinquefasciatus* collected from a sewage pond adjacent to a residential area were shown to have fed on an avian host, compared with 73% of females collected from a swamp adjacent to an agricultural area (Edman 1974). Although other host range studies in the southern United States have observed a high level of ornithophagic behaviour in *Cx. quinquefasciatus* (Bertsch and Norment 1983, Irby and Apperson 1988), a greater number of host range studies on North American populations have reported this species to feed primarily on mammalian hosts. Avian species represented a minority of hosts identified from blood-fed *Cx. quinquefasciatus* collected from, and adjacent to, residential areas in Florida, California, Arizona, and Texas (Edman 1974, Reisen *et al.* 1990, Zinser *et al.* 2004, Molaei *et al.* 2007).

Previous host preference studies have demonstrated a strong affinity of *Cx. quinquefasciatus* for canine hosts (Loftin *et al.* 1997, Labarthe *et al.* 1998). In East Baton Rouge Parish, a previous host range study identified the domestic dog as the most common host of *Cx. quinquefasciatus* (Niebylski and Meek 1992). Canine blood was detected in over two-thirds of blood-fed *Cx. quinquefasciatus* collected from residential neighborhoods, and approximately a third of females collected from adjacent hardwood forests. The domestic dog also has been identified as the primary host of *Cx. quinquefasciatus* in Texas (Molaei *et al.* 2007), and the most common mammalian host in Hawaii, southern California and Florida (Hess and Hayes 1970,

Edman 1974, Reisen *et al.* 1990). The host preference for dogs may reflect the very close association of *Cx. quinquefasciatus* with suburban and urban development (Schreiber *et al.* 1989, Reisen *et al.* 1992, Savage *et al.* 2006). In southern Louisiana, *Cx. quinquefasciatus* and *Cx. salinarius* are two of the most frequently collected mosquito species around homes in suburban areas (Collier *et al.* 2006). In the current study, although the domestic dog represented less than ten percent of all identified *Cx. quinquefasciatus* bloodmeals, it was the second most frequent mammalian host, and about half of all mammalian bloodmeals were obtained from domestic species. This domestic association is supported by the high degree of anthropophagy observed. Although the proportion of bloodmeals obtained from mammalian hosts was significantly lower in *Cx. quinquefasciatus,* this species fed much more frequently on human hosts than mosquitoes of the other three *Culex* species examined.

The overall rate of anthropophagy observed in our study (7%) was less than previously reported for *Cx. quinquefasciatus* in EBRP. Niebylski and Meek (1992) found that between 11 and 15 percent of bloodfed females collected from residential areas, and between 16 and 23 percent of bloodfed *Cx. quinquefasciatus* females collected from adjacent hardwood forests, had fed on a human host. A low rate of human feeding (0.4 to 1.5%) has been reported in *Cx. quinquefasciatus* collected from urban and suburban locations Harris County,Texas (Kokernot *et al.* 1974, Molaei *et al.* 2007). A similar low human feeding rate (<0.5%) was detected in *Cx. quinquefasciatus* collected from more rural locations in Mississippi (Bertsch and Norment 1983). Molaei *et al.* (2007) suggested that the low rate of anthropophagy observed in Harris County, TX, may have been due to high temperatures inhibiting human outdoor activities during the summer months. However, high human feeding rates (>25%) have been reported in *Cx.*

quinquefasaciatus collected from urban habitats in Arizona, Mexico and Brazil (Gomes *et al.* 2003, Zinser *et al.* 2004, Elizondo-Quiroga *et al.* 2006).

The moderate competence for transmitting WNV (Sardelis *et al.* 2001, Goddard *et al.* 2002), the relatively high rate of infection, and high rate of blood feeding on passerine hosts are consistent with *Cx. quinquefasciatus* acting as the primary enzootic vector of WNV in southern Louisiana (Godsey *et al.* 2005b, Gleiser *et al.* 2007). The significant rate of feeding on humans and domestic animals suggests that it is also probably very important as a bridge vector in residential areas.

In the current study, we did not detect a significant seasonal shift from feeding primarily on birds in the spring and early summer, to feeding primarily on mammals in the late summer and early fall, for *Cx. nigripalpus* or *Cx. quinquefasciatus*. Niebylski and Meek (1992) also were unable to observe a seasonal host shift in the feeding behavior of *Cx. quinquefasciatus* in EBRP. The absence of a host shift may have been partly due to the wide variability in the host fauna among sites. Among sites from which a total of at least twenty blood-fed *Cx. quinquefasciatus* were collected, the percentage of mammalian feeding ranged from 17.4 percent in the collections from Highland Road, to 50 percent in the Ednie Lane collections (data not shown). Pooling data among 17 sites may have masked changes in the host utilization occurring at individual sites. However, our data indicates that a host shift is not required for epidemic transmission of WNV to occur in EBRP. Both *Cx. quinquefasciatus* and *Cx. nigripalpus* readily fed on both competent reservoir hosts (passeriform birds) and incidental hosts (humans and equines) throughout the summer and fall when the majority of human and equine cases are reported.

In this study, the blood of multiple host species was detected in 0.9 percent of the identified *Cx. quinquefasciatus* bloodmeals, 1.8 percent of the identified *Cx. nigripalpus*

bloodmeals, and 3.1 percent of the identified *Cx. salinarius* bloodmeals. Other studies have reported comparable multiple host bloodfeeding rates. Blood from more than one host species was detected in approximately 4.8 percent of bloodfed *Cx. quinquefasciatus* collected from a residential area in Arizona (Zinser *et al.* 2004). Only 0.1 percent of bloodfed *Cx. salinarius* collected from saltmarshes in southwestern Louisiana reacted with antisera to more than one host group (Schaefer and Steelman 1969). Multiple host bloodmeals have been detected in less than one percent of *Cx. nigripalpus* collected from Florida, Panama and Brazil (Edman 1974, Christensen *et al.* 1996, Gomes *et al.* 2003). In a previous study in EBRP, none of the bloodfed *Cx. quinquefasciatus* collected were reported to have fed on more than one host species (Niebylski and Meek 1992). Other studies also have failed to detect multiple host bloodmeals in *Cx. quinquefasciatus* and *Cx. salinarius* (Suyemoto *et al.* 1973, Irby and Apperson 1988, Gomes *et al.* 2003).

A number of studies have reported much higher rates of multiple host bloodfeeding than observed in the current study. In *Cx. salinarius*, multiple feeding rates of 13 percent or higher have been reported (Cupp and Stokes 1976, Gingrich and Williams 2005). Edman and Downe (1964) detected blood from multiple host species in 36.7 percent of bloodfed *Cx. salinarius* collected from Kansas. Similarly, sequencing of host *cytb* demonstrated that 8 percent of *Cx. quinquefasciatus* collected from Texas, and 11 percent of *Cx. salinarius* collected from Connecticut, contained both mammal and avian DNA (Molaei *et al.* 2006, Molaei *et al.* 2007).

In this study, the Northern Raccoon was the most frequently identified host of *Cx. nigripalpus*, and the second most frequently identified host of *Cx. quinquefasciatus* and *Cx. salinarius*. Abundant food resources in urban and suburban habitats can allow the Northern Raccoon to reach very high densities in urban and suburban habitats (Prange *et al.* 2003, Prange

et al. 2004). The Virginia Opossum, *Didelphis virginiana*, was the third most frequent mammalian host of *Cx. nigripalpus*, and the fourth most frequent mammalian host of *Cx. quinquefasciatus*. The Virginia Opossum also responds positively to habitat fragmentation and is tolerant of urbanization (Crooks 2002). Both host species are very abundant in East Baton Rouge Parish (Lowery 1974).

The high feeding frequency of *Culex* species on Northern Raccoons and Virginia Opossums may be a result of both host density and host behavior. Caged host studies have demonstrated that Northern Raccoons and adult Virginia Opossums exhibit relatively weak defensive behavior in response to feeding *Cx. nigripalpus* allowing a high feeding success rate (Edman *et al.* 1974). Also, the activity patterns of both host species corresponds to the time of day when host seeking *Cx. quinquefasciatus*, *Cx. nigripalpus* and *Cx. salinarius* are most active (Provost 1969, Lowery 1974, Anderson and Hudson 1980, Slaff and Crans 1981, Meyer *et al.* 1984).

The high rate of feeding by *Culex* mosquitoes on wild, mammalian mesopredators is consistent with WNV seropositive rates observed in southern Louisiana. In 2002, a WNV outbreak occurred in St. Tammany Parish, Louisiana, with high rates of infection reported in humans, mosquitoes and sentinel chickens (Balsamo *et al.* 2003, Palmisano *et al.* 2005). Approximately 75 percent of Virginia Opossums and 60 percent of Northern Raccoons collected in 2002 from Slidell, a community in the southeastern corner of St. Tammany Parish, were positive for WNV-specific antibodies (Dietrich *et al*. 2005). In 2003, specific antibodies to WNV also were detected in about 10 percent of Northern Raccoons and 17 percent of Virginia Opossums collected from Ouachita and Calcasieu Parishes, Louisiana (Bentler *et al.* 2007). Evidence of WNV infection also has been detected in raccoons sampled in Wisconsin,

Pennsylvania, New York and Wyoming, and in opossums sampled in Wisconsin, New York, Ohio, Pennsylvania, Texas and Wyoming (CDC 2000, Root *et al.* 2005, Docherty *et al.* 2006, Bentler *et al.* 2007). Although oral transmission of WNV to mesopredator species feeding on infected prey may increase their exposure (Root *et al.* 2005), the results of the current study suggest that vector-borne transmission of WNV alone could support the high seropositive rates observed in Northern Raccoons and Virginia Opossums in southern Louisiana.

The White-tailed Deer is a very abundant large vertebrate species in East Baton Rouge Parish (Lowery 1974), and prefers more fragmented woodlots. Increased density of edge habitats in suburban areas can allow deer to reach high densities (Gaughan and DeStaefano 2005). White-tailed Deer also is more tolerant of mosquito feeding than smaller mammal hosts (Nasci 1984). In the current study, the White-tailed Deer was a frequent source of blood for *Cx. coronator, Cx. salinarius* and *Cx. nigripalpus,* but not for *Cx. quinquefasciatus*. In Iowa, neutralizing antibodies to WNV were detected in about eight percent of White-tailed Deer tested in the first two years after the virus was first detected in the state (Santaella *et al.* 2005).

Most mammal species evaluated as amplifying hosts, including domestic horses, cats, dogs and pigs, have been demonstrated to be refractory or very weakly competent for WNV (Bunning *et al.* 2002, Austgen *et al.* 2004, Teehee *et al.* 2005). Several domestic mammalian species were frequently fed on by *Culex* mosquitoes in the current study. Domestic dogs and cats were particularly important mammalian hosts of *Cx. salinarius* and *Cx. quinquefasciatus*. Abundant domestic hosts in urban and suburban habitats could be zooprophylactic by reducing the rate of blood feeding on competent reservoir hosts by *Culex* vectors, as suggested by Hess and Hayes (1970). The reservoir competences of the Northern Raccoon, Virginia Opossum and White-tailed Deer for WNV have not been examined, although both of the former species have

been demonstrated to be poor amplifying hosts of SLEV and LACV (Amundson *et al.* 1985, McClean *et al.* 1985).

Reservoir competence for WNV has been demonstrated in a small number of rodent and lagomorph species. Eastern Cottontail Rabbits infected after exposure to infected mosquitoes can develop a sufficient level of circulating virus to infect *Cx. salinarius* and *Cx. pipiens* (Tiawsirisup *et al.* 2005). California Fox Squirrels, Eastern Chipmunks, and Golden Hamsters may develop a WNV viremia of 5 $\log_{10}$PFU / ml or higher (Xiao *et al.* 2001, Padgett *et al.* 2007, Platt *et al.* 2007), which is sufficient to infect competent mosquito vector species. Although the reservoir competence of most native rodent and lagomorph species in Louisiana for WNV is unknown, low feeding rates by important *Culex* vectors would limit the role of these vertebrate species as amplifying hosts of the virus. Though many small mammal species are abundant in urban habitats in East Baton Rouge Parish (Lowery 1974), very few bloodmeals from rodent and lagomorph hosts were detected in blood-fed *Cx. quinquefasciatus* and *Cx. salinarius* in the current study, and none were detected in *Cx. coronator* or *Cx. nigripalpus*. This may be largely a function of host behavior rather than low host abundance, as suggested by the low rate of WNV exposure observed in small rodent species in southern Louisiana. Although WNV seroprevalence in mesopredator species collected from Slidell during the WNV outbreak in 2002 was greater than 50 percent, neutralizing antibodies to WNV were detected in only six percent of Roof Rats, four percent of Hispid Cotton Rats, and two percent of Eastern Gray Squirrels (Dietrich *et al.* 2005). In caged host studies, Edman *et al.* (1974) observed a very strong defensive response in several small rodent species exposed to *Cx. nigripalpus*, which greatly reduced blood feeding success.

A two year serosurvey of WNV neutralizing antibodies in wild birds was examined concurrently at many of the same East Baton Rouge Parish study sites sampled in our study (Gruszynski 2006). Seroprevalence in many of these avian hosts was consistent with the relative rate of exposure to blood feeding *Cx. quinquefasciatus* observed in the current study. The overall proportion of birds collected by mist netting over the two year period positive of neutralizing antibodies for WNV was 25.7 percent in Northern Cardinals, 40 percent in Northern Mockingbirds, 34.8 percent in Mourning Doves, 19.6 percent in House Sparrows, 19.1 percent in Brown Thrashers, and 14.3 percent in Blue Jays. Exposure to the virus likely occurred in East Baton Rouge Parish; all six species are permanent residents of southern Louisiana, although many Brown Thrashers migrate to Louisiana from northern states in the spring (Lowery 1974). Similar WNV seroprevalence rates were measured in Northern Cardinals (48%), Northern Mockingbirds (50%), Mourning Doves (21.4%), House Sparrows (20.2%), and Blue Jays (30%), collected from a residential neighborhood in St. Tammany Parish during the WNV outbreak in 2002 (Komar *et al.* 2005). High WNV seroprevalence rates have also been observed in Northern Cardinals (75%) and Northern Mockingbirds (50%) collected from northern Florida, although the number of birds sampled was small (Godsey *et al.* 2005a). West Nile virus antibody seroprevalence rates were 27.4 percent in Northern Cardinals, 23.2 percent in Northern Mockingbirds, 17.7 percent in Mourning Doves, 19.3 percent in Blue Jays, but only 8.0 percent in House Sparrows, in a survey of live, mist-netted birds from Harris County, Texas, (Molaei *et al.* 2007).

In a few avian species from East Baton Rouge Parish, the WNV seroprevalence reported by Gruszynski (2006) was less than expected, based on the rate of feeding by *Cx. quinquefasciatus* in the current study, including 16.7 percent in Common Grackles, and 2.8

percent in Tufted Titmice. This is similar to the 9.1 percent of the Common Grackles sampled in St. Tammany Parish, and 15.4% of the Gommon Grackles tested in Florida, that were seropositive for WNV antibodies (Godsey *et al.* 2005a, Komar *et al.* 2005). Although Komar *et al.* (2003a) reported a 33.3 percent mortality rate in Common Grackles exposed to WNV in the laboratory, this probably does not account for the lower than expected seroprevalence rate. Komar *et al.* (2003a) observed even higher mortality rates in Blue Jays (75%) and House Sparrows (50%).

In the current study, Carolina Wrens were fed on by *Cx. quinquefasciatus* at a rate lower than expected based on their abundance and proportion of the total avian biomass. The seroprevalence rates for Carolina Wrens observed in East Baton Rouge Parish (16.7%; Gruszynski 2006) and St. Tammany Parish (35.3%; Komar *et al.* 2005) suggest that contact with infected vectors is much more frequent than suggested by the results of the current study. Similarly, Molaei *et al.* (2007) detected a relatively high WNV seroprevalence (18.7%) in Carolina Wrens Harris County, Texas, but identified this species from only a very small proportion (0.3%) of *Cx. quinquefasciatus* bloodmeals. This suggests that exposure of Carolina Wrens to WNV may be due to a vector other than *Cx. quinquefasciatus*, or that *Cx. quinquefasciatus* is feeding on Carolina Wrens in a habitat not sampled in the current study.

The most frequent avian host of *Cx. quinquefasciatus* and *Cx. nigripalpus*, and the third most abundant avian species in the NABBS counts in 2003 and 2004, was the Northern Cardinal. Northern Cardinal density is positively correlated with the density of edge habitat (Brennan and Schnell 2005), which may allow it to adapt well to urbanization. Experimentally infected Northern Cardinals can develop a peak viremia greater than 10^8 PFU / ml of serum (Komar *et al.* 2005), sufficient to infect susceptible vector species, including *Cx. quinquefasciatus* (Sardelis *et*

al. 2001). Based on the abundance of this species in East Baton Rouge Parish, its close association with residential areas, its importance as a host of *Culex* vectors throughout most of the year, and its susceptibility to WNV infection, the Northern Cardinal may be the most important amplifying host of WNV in urban habitats of southern Louisiana.

The Northern Mockingbird was the second most frequent avian host of *Cx. quinquefasciatus,* and the fourth most abundant avian species in the NABBS counts in 2003 and 2004. This species also is well adapted to residential habitats (Blair 1996). Experimental infections in Northern Mockingbirds indicate that this species is a moderately competent reservoir of WNV; developing an average peak viremia of approximately 10^6 PFU / ml of serum (Komar *et al.* 2005). Northern Mockingbirds are likely to be important reservoir hosts of WNV in urban areas in southern Louisiana.

The Common Grackle was the third most common avian host of *Cx. quinquefasciatus,* and second most common avian host of *Cx. nigripalpus*. This species is abundant throughout most parts of Louisiana; nesting and foraging in large groups (Yang and Selander 1968). This species is considered a highly competent reservoir host for WNV (Komar *et al*. 2003a), and may be an important reservoir host of WNV, although the serological evidence suggests only a moderate WNV infection rate in EBRP. The Common grackle may be involved in early season amplification and overwintering maintenance of WNV; this species was particularly important host of *Cx. quinquefasciatus* in the spring, and both *Cx. quinquefasciatus* and *Cx. nigripalpus* in the late fall.

Mourning Doves can be found in a wide variety of urban areas; from suburban residential habitats, to more open and developed habitats (Blair 1996). In the current study, this species was the fourth most frequent avian host of *Cx. quinquefasciatus*. Mourning Doves are only weakly

competent reservoirs of WNV (Komar *et al.* 2003a, Reisen *et al.* 2005), and may not contribute significantly to the enzootic amplification of WNV.

In the current study, the domestic chicken was a significant source of blood for *Cx. quinquefasciatus, Cx. nigripalpus* and *Cx. salinarius*. These results may have been skewed due to the trapping site selection. Almost all of the mosquitoes containing chicken blood were collected from sites where EBRPMARC maintained a sentinel chicken within 200 m of the trapping location, or from a residential site with numerous, free-ranging poultry (Denham Road). The low abundance of chickens in residential areas, and poor reservoir competence for WNV (Langevin *et al.* 2001), would preclude chickens as important to the enzootic amplification of WNV in urban areas of southern Louisiana.

House Sparrows were fed on by *Cx. quinquefasciatus* less frequently than expected in the current study, based on their abundance, and a moderately high WNV seroprevalence rate in East Baton Rouge Parish (Gruszynski 2006). However this species is a relatively competent reservoir host for WNV (Komar *et al.* 2003a), is very closely associated with urban habitats (Blair 1996), and is very abundant in East Baton Rouge Parish. The House Sparrow is likely an important reservoir host for urban WNV transmission in southern Louisiana.

Members of the avian family Corvidae have been demonstrated to be particularly susceptible to infection with WNV (Komar *et al.* 2003a), and exposure rates in live and dead corvids are often used to detect early season WNV activity (Eidson *et al.* 2001, Lindsay *et al.* 2003). Many corvid species can acquire the virus orally by preying or scavenging on infected birds (Komar *et al.* 2003a). The Blue Jay is one of the most common corvid species in urban areas of East Baton Rouge Parish, and has been demonstrated in the laboratory to be an extremely competent reservoir host for WNV (Komar *et al.* 2003a). Komar *et al.* (2003a)

recorded a peak WNV viremia of $10^{12.1}$ PFU / ml of serum in experimentally infected Blue Jays, which is sufficient to infect vector species with a lower susceptibility to infection. Our data suggests that this species is in one of the most common hosts of *Cx. quinquefasciatus* during the winter months. This is consistent with the findings of a dead bird surveillance program in Harris County, Texas, where dead Blue Jays yielded the greatest number of WNV positive samples from January, 2003, to March, 2004 (Tesh *et al.* 2004). The Blue Jay is relatively abundant in EBRP, and may be an important amplifying host, especially during the cooler months of the year.

The two other common corvid species in East Baton Rouge Parish are the American Crow and the Fish Crow. In urban areas in southern California, WNV infection in humans and *Cx. quinquefasciatus* is significantly correlated with American Crow density and WNV infection (Reisen *et al.* 2006d). The American Crow also may be very important in the dispersal of WNV in urban environments. American Crows are considered highly competent reservoir hosts of WNV (Komar *et al.* 2003a), and move great distances between roosts (Ward *et al.* 2006). In the current study, no bloodmeals from the American Crow were detected in the four *Culex* species examined. This is consistent with the observations made on the host ranges of *Cx. quinquefasciatus* in Texas, and *Cx. pipiens* in the northeastern United States; both vector species were found to rarely feed on American Crows (Apperson *et al.* 2002, Apperson *et al.* 2004, Molaei *et al.* 2006, Molaei *et al.* 2007). We did detect Fish Crow DNA in a small number of blood-fed *Cx. quinquefasciatus* and *Cx. nigripalpus*, but the FR for *Cx. quinquefasciatus* was significantly less than one, indicating that Fish Crows are not a preferred host. Although Fish Crows are not frequently fed on by *Culex* vectors, and are only a moderately competent reservoir for WNV (Komar *et al.* 2003a), they may be frequently exposed the virus through an oral route.

In Florida citrus groves, Fish Crows are one of the primary predators of nestling Brown Thrashers, Red-winged Blackbirds and Northern Cardinals (Mitchell *et al.* 1996), three species with high WNV seroprevalence rates in EBRP.

In the current study, avian hosts with the largest and smallest average body mass were fed on less frequently than expected, based on their estimated abundance. The smallest size class (<30 g) includes some very common songbirds, including several warbler and wren species. The FR of one of the most common species from this size class in the parish, the Carolina Wren, was significantly less than one, even when adjusted for biomass.

Almost half of birds in the largest avian size class are represented by ciconiiform and anseriform species primarily associated with wetland habitats (Lowery 1974). In the current study, although many of the sites sampled were located close to drainage ditches, only two were adjacent to large, permanent bodies of water. The other large avian hosts were falconiiform and large corvid (Fish Crow and American Crow) species. These species nest and roost fairly high in the canopy (Gough *et al.* 1998), and may be encountered by host seeking *Culex* less frequently than avian hosts that spend more time at lower heights.

In this study, we examined the host-utilization patterns of four species of *Culex* mosquitoes common in EBRP. *Culex quinquefasciatus* fed more frequently on domestic hosts and host species closely associated with human habitation. Both *Cx. nigripalpus* and *Cx. coronator* fed more frequently on host species more closely associated with undeveloped areas. The host feeding pattern of *Cx. salinarius* was intermediate between these two strategies.

Multiple permanent resident, passerine species were important hosts of *Cx. nigripalpus* and *Cx. quinquefasciatus*, including the Northern Cardinal, the Northern Mockingbird, the Common Grackle, the House Sparrow, and the Blue Jay. Epizootic amplification of WNV in

East Baton Rouge Parish is likely maintained in these host species during the late spring, summer, and early fall months by *Cx. quinquefasciatus*. In the late summer and fall, *Cx. nigripalpus* may also contribute to enzootic transmission. The utilization of Blue Jays, Northern Cardinals and Common Grackles by *Cx. quinquefasciatus* during the winter and spring also can be important for maintaining year round enzootic transmission of WNV in EBRP. These may be common features of WNV enzootic transmission in suburban and urban areas of the southeastern United States. Very similar WNV epizootiology, involving the same reservoir hosts and *Cx. quinquefasciatus*, has been described in St. Tammany Parish, Louisiana (Godsey *et al.* 2005b), as well as northern Florida and southern Georgia (Godsey *et al.* 2005a).

Human DNA was detected in a significant proportion of blood-fed *Cx. quinquefasciatus*, and small proportion of blood-fed *Cx. nigripalpus*. Although the *Cx. salinarius* and *Cx. coronator* collected in this study fed primarily on mammalian hosts, no human bloodmeals were detected in either species. The higher rate of anthropophagy in *Cx. quinquefasciatus* is consistent with its preference for feeding on avian species associated with suburban habitats, and domestic mammals.

In addition to the four *Culex* species examined, WNV has been detected in specimens of at least 15 mosquito species collected from EBRP (Chapter 2, Tables 2.2 to 2.4). Two of these species, *Aedes albopictus* (Skuse) and *Ochlerotatus triseriatus* (Say), were ranked by Turell *et al.* (2005) as potential WNV bridge vectors of moderate importance. Both of these species will breed in artificial container habitats associated with residential homes, are active during the daytime, will feed on human hosts, and are at least moderately competent vectors of WNV (Chapman and Johnson 1986, Turell *et al.* 2005, Turell *et al.* 2001, Richards *et al.* 2006). However in EBRP, *Cx. quinquefasciatus* is far more abundant in most habitats, has a wider

seasonal and spatial distribution, and has a much greater WNV infection rate, than either *Ae. albopictus* or *Oc. triseriatus* (Chapter 2, Table 2.2 to Table 2.4).

As well as serving as the primary enzootic vector of WNV in EBRP, *Culex quinquefasciatus* is also likely the primary epidemic vector. *Culex nigripalpus* may be important as a late season bridge vector, whereas *Cx. salinarius* may serve more as a bridge vector to equines and other domestic animals. The vector competence of *Cx. coronator* is unknown, but the host range data suggests that it is a potential bridge vector to large mammals, such as equines.

CHAPTER 4. SEASONAL PREVALENCE OF WEST NILE VIRUS IN MALE MOSQUITOES IN EAST BATON ROUGE PARISH, LOUISIANA

4.1 Introduction

Horizontal transmission of an arbovirus to a female mosquito can occur when the virus is acquired orally during bloodfeeding on an infective vertebrate host, or venereally during copulation via seminal fluid obtained from an infected male mosquito. Arboviruses also may be transmitted vertically from an infected female to her progeny. Both vertical and venereal transmission in mosquito vectors has been demonstrated for several flaviviruses, including St. Louis Encephalitis virus (Hardy *et al.* 1980, Nayar *et al.* 1986), Yellow Fever virus (Aitken *et al.* 1979), Dengue virus (Rosen *et al.* 1983, Rosen 1987a), Japanese Encephalitis virus (Rosen *et al.* 1978, Rosen *et al.* 1989), and West Nile virus (Baqar *et al.* 1993, Reisen *et al.* 2006c). These transmission pathways may enhance arbovirus survival during periods when horizontal transmission between arthropod vectors and vertebrate hosts is limited.

The first field evidence of vertical transmission of WNV was the isolation of virus from a pool containing four male mosquitoes of the *Cx. univittatus* complex that had been collected from western Kenya (Miller *et al.* 2000). Since then, a significant amount of evidence of vertical transmission of WNV in natural vector populations in North America has been accumulated. In the northeastern United States, WNV has been detected in male *Cx. pipiens* Linnaeus, in nulliparous and/or overwintering female *Cx. pipiens*, and the progeny of naturally infected, overwintering female *Cx. pipiens* (Nasci *et al.* 2001, Bugbee and Forte 2004, Farajollahi *et al.* 2005, Anderson *et al.* 2006, Anderson *et al.* 2007). However, other attempts to detect WNV in overwintering female *Cx. pipiens* in Colorado, and male *Cx. pipiens* collected from Ohio during the summer, were unsuccessful (White *et al.* 2006, Bolling *et al.* 2007). Reisen *et al.* (2006c)

failed to detect WNV in diapausing female *Cx. tarsalis* Coquillett collected from overwintering sites in southern California, but were able to detect virus in male *Cx. quinquefasciatus* that had been collected from gravid traps, and in adult male *Cx. quinquefasciatus* reared from egg rafts collected from the field, during the summer months. West Nile virus also has been detected in field collected larvae of *Cx. erythrothorax* Dyar collected from Utah (Phillips and Christensen 2006).

Male mosquitoes are unlikely to acquire WNV horizontally; males do not ingest vertebrate blood, and laboratory experiments with La Crosse virus (LACV) have failed to demonstrate any evidence that male mosquitoes could acquire the virus during copulation with infected females (Thompson and Beaty 1978). Although male *Culex quinquefasciatus* Say can become infected with St. Louis Encephalitis virus (SLEV) by ingesting the virus as larvae under laboratory conditions (Collins 1963), these experiments involved incubating the larvae in high titre virus suspensions ($> 10^5$ mouse IC LD_{50} per ml), a situation that is unlikely to occur in nature. Similar experiments conducted with high titre suspensions of WNV failed to infect larval *Cx. tritaeniorhynchus* Giles (Baqar *et al*. 1993). Therefore, the only route a male mosquito is likely to naturally acquire WNV is vertically from an infected female parent. Since there appears to be no difference between the filial infection rates of male progeny and the filial infection rates of female progeny (Reisen *et al*. 2006c, Baqar *et al.* 1993), infection rates in field collected male mosquitoes may be considered both as an indication of the potential for venereal transmission, and as a direct estimate of the vertical transmission rate in the female mosquito population.

In Louisiana, the intensity of enzootic WNV transmission is highest during the summer and early fall (Palmisano *et al.* 2005, Gleiser *et al.* 2007). The overwintering maintenance of WNV prior to enzootic amplification in the spring has not been well described in Louisiana.

Although a low level of transmission between *Culex* mosquito vectors and avian reservoir hosts is thought to be maintained throughout the year in Louisiana (Tesh *et al.* 2004), maintenance of the virus solely within the vector population also could be an important alternative overwintering strategy for WNV. Vertical and venereal transmission mechanisms also could enhance epizootic and epidemic WNV transmission in the spring and summer since nulliparous female mosquitoes would be infective.

Surveillance of WNV activity in vector populations primarily involves the collection and testing of female mosquitoes for virus. Since 1999, evidence of WNV infection has been documented in females of at least 62 mosquito species in North America (CDC 2007e). However, few attempts have been made to determine if WNV can be detected in field collected, adult male mosquitoes (Anderson *et al.* 2006, Reisen *et al.* 2006c, White *et al.* 2006). The objective of this study was to test the males of several mosquito species collected from East Baton Rouge Parish (EBRP) for presence of WNV RNA. This information could be useful for evaluating the potential for vertical and venereal transmission of WNV by different mosquito species.

4.2 Materials and Methods

The first male samples were collected from 17 sites in the northern half of the parish by EBRPMARC personnel (see Appendix C). These were the same sites sampled by EBRPMARC to determine WNV infection in female mosquitoes in an earlier study (Chapter 2). The EBRMARC samples were collected over two consecutive 24 hour periods, every two weeks, from November 2002 to October 2003, as described in chapter two.

Male mosquitoes also were collected by LSU Agcenter personnel from the same sites in East Baton Rouge parish, East Feliciana Parish, and Iberville Parish, that were sampled

(sampling was performed as described in chapter 3) for bloodfed mosquitoes (Table 3.1, see Appendix C). Samples were collected from November, 2002, to December, 2003. In 2004, samples were only collected in March.

Mosquitoes were sorted by species on a chill table (BioQuip®, Gardena, CA) using a stereo dissecting microscope (M5-65508; Wild Herrbrugg, Switzerland). Male mosquitoes were identified based on morphological characters using Darsie and Ward (1981) and Stojanovich (1960). Males were initially pooled by species, by site and by date of collection, and placed into sterile, 2mL Costar® microcentrifuge tubes (Cat. No. 3213, Corning Inc., Corning, NY) containing a maximum of 50 males per pool. As a control for cross-contamination of specimens with WNV, non-hematophagous arthropods collected in the traps, mostly chironomidae, psychodinae and aphidoidea specimens, also were tested for WNV.

Males collected by EBRPMARC personnel were submitted to the LSU Veterinary School Diagnostic Laboratory for WNV detection by virus isolation. The methods used are described in chapter two.

Males collected by LSU Agcenter personnel were tested for the presence of WNV RNA using Real Time reverse transcriptase polymerase chain reaction (RT-PCR). Male collections were further consolidated by species and date range to reduce the number of pools tested. A standard curve was used to estimate the amount of virus present in each sample. To construct the standard curve, a serial dilution was made using virus stock diluted 1:10 in BA-1 media. Volumes of 225 µl of each of the concentrations: 4×10^6, 4×10^4, 4×10^2, 4×10^1, and 4×10^0 PFU / ml, were added to 2.0 ml microcentrifuge tubes containing 75, 50, 25, 10 or 0 *Cx. quinquefasciatus* mosquitoes. Mosquitoes used for the standard curve were of the Sehbring strain of *Cx. quinquefasciatus*, and were obtained from the Harris County Mosquito Control Division,

where they had been maintained in culture since 1995 (Pam Stark *personal communication).* An additional negative control, and PCR and extraction positive controls, were included in each extraction. RNA extractions were performed using the RNeasy kit (Qiagen, Valencia, CA) as described in Chapter two. Field collected samples were grouped by pool size (>60 mosquitoes, 35-59 mosquitoes, 20-34 mosquitoes, and <20 mosquitoes). For each 96 column plate, RNA was extracted from two standard curves in duplicate. The first set of standards contained the five virus concentrations and no mosquito tissue, and the second set of standards contained homogenates of the five virus concentrations and uninfected *Cx. quinquefasciatus* mosquitoes; the number of mosquitoes in the second standard was dependent on the size of the samples. Samples containing 60 or more males were processed on the same column plate as the standard containing 75 uninfected *Cx. quinquefasciatus* mosquitoes, samples containing 35-59 males were processed on the same column plate as the standard containing 50 uninfected *Cx. quinquefasciatus* mosquitoes, samples containing 20-34 males were processed on the same column plate as the standard containing 25 uninfected *Cx. quinquefasciatus* mosquitoes, and samples containing less than 20 males were processed on the same column plate as the standard containing 10 uninfected *Cx. quinquefasciatus* mosquitoes. The procedures used for RT-PCR are described in chapter two. Samples that produced a level of florescence above the critical threshold within 40 cycles were considered positive for the presence of WNV RNA.

4.3 Results

A total of 171 male mosquitoes, of at least 12 species, were grouped into 45 pools and submitted by EBRPMARC for WNV detection by virus isolation (Table 4.1). Almost two-thirds (64.3%) of the males submitted for WNV detection by the virus isolation assay were collected from March through June 2003. Few males (n=24) collected by EBRPMARC personnel after

June were submitted. West Nile virus was isolated from only 2 pools; a pool containing two male *Cx. salinarius* collected on 22 April, 2003, and a pool containing a single male *Oc. triseriatus* Say collected 25 April, 2003.

Table 4. 1. Isolation of WNV from male mosquitoes collected from East Baton Rouge Parish, November, 2002 to October, 2003.

Species	No. Males	No. Pools	Date of Collection of WNV+ Pool (No. WNV+ Pools)
Culex quinquefasciatus	63	10	
Aedes vexans	59	12	
Anopheles crucians	19	9	
Psorophora howardii	7	1	
Cx. salinarius	5	2	22 April 2003 (1)
Uranotaenia lowii Theobald	5	2	
Ur. spp.	3	2	
An. quadrimaculatus Say	3	1	
Ps. ciliata (Fabricius)	2	1	
Cx. nigripalpus	1	1	
Ochlerotatus spp.	1	1	
Oc. triseriatus	1	1	25 April 2003 (1)
Ps. ferox	1	1	
Ur. sapphirina (Osten Sacken)	1	1	

One hundred-forty eight pools, containing a total of 7230 male mosquitoes of at least 15 species, were tested for the presence of WNV RNA (Table 4.2). Four pools were considered positive for WNV; single pools containing *Ps. howardii* (Coquillett) and *An. crucians* Wiedemann males collected in late March 2003, a pool of *Cx. restuans* collected in May 2003, and a pool containing *Oc. triseriatus* collected from March to November 2003. The total numbers of male *Cx. restuans*, *Oc. triseriatus*, *Ps. howardii* and *An. crucians* collected per month and submitted for WNV RNA testing by RT-PCR are shown in Figure 4.1. West Nile virus RNA was not detected in the two pools of non-hematophagous arthropods included as negative controls.

Table 4. 2. Detection of WNV from male mosquitoes collected from sites in and adjacent to East Baton Rouge Parish, November, 2002 to March, 2004.

Species	No. Males	No. Pools	Collection Date of WNV+ Pools (No. +ve Pools)	Ct value	Estimated WNV Conc. (PFU / ml)[2]
Culex quinquefasciatus	3899	73			
Aedes vexans	1177	25			
Uranotaenia sapphirina	521	7			
Cx. species[1]	380	8			
Cx. territans Walker	225	5			
Cx. erraticus	185	4			
Cx. restuans	142	5	7-15 May 2003 (1)	40.0	$<4 \times 10^6$
Psorophora howardii	140	4	27 March 2004 (1)	38.8	$<4 \times 10^6$
Anopheles crucians	128	3	27-31 March 2003 (1)	39.0	4×10^1 - 4×10^6
Ae. albopictus	118	4			
Cx. salinarius	116	3			
Ochlerotatus canadensis	58	2			
Cx. nigripalpus	46	2			
Oc. triseriatus	46	1	26 March to 06 November 2003 (1)	38.8	4×10^4 - 4×10^6
An. quadrimaculatus	45	1			
Ur. lowii	4	1			
Non-hematophagous arthropods (negative controls)	39	2			

[1] Not identified to species prior to testing by RT-PCR; either *Cx. restuans* or *Cx. quinquefasciatus.*

[2] Number of plaque forming units per ml estimated from Ct values of standards containing a similar number of uninfected *Cx. quinquefasciatus* and known concentrations of WNV.

For each pool size, WNV RNA was detected in an insufficient number of known standards to allow calculation of a regression line. For the 4×10^6 PFU / ml concentration of WNV, the average Ct values of the two replicates were 33.11 for the 75 mosquito pool size, 35.13 for the 50 mosquito pool size, 31.94 for the 25 mosquito pool size, and 28.12 for the 10 mosquito pool size. The average Ct value for the 4×10^4 PFU / ml concentration containing 10 mosquitoes was 35.63. West Nile virus RNA was detected in single replicates of the 4×10^4 PFU / ml concentration containing 50 mosquitoes (Ct = 39.03), and the 4×10^1 PFU / ml concentration containing 25 mosquitoes (Ct = 39.50).

For the standards containing no mosquitoes, the mean Ct values were 28.18 ± 0.38 for the 4 x 10^6 PFU / ml concentration and 34.68 ± 1.97 for the 4 x 10^4 PFU / ml concentration (n=8). An average Ct value of 38.83 was recorded for the 4 x 10^2 PFU / ml concentration containing no mosquitoes (n=2), and WNV was detected in a single replicate of the 4 x 10^1 PFU / ml concentration containing no mosquitoes (Ct = 38.81). We failed to detect WNV RNA in any of the other standards.

4.4 Discussion

In the current study, WNV RNA was detected in single pools containing male *Cx. restuans, Oc. triseriatus, Ps. howardii* and *An. crucians*. West Nile virus also was isolated from a pool containing male *Cx. salinarius* and a pool containing male *Oc. triseriatus*. Previous studies have detected WNV or WNV RNA in field collected male *Cx. pipiens* (Farajollahi *et al.* 2005, Anderson *et al.* 2006) and *Cx. quinquefasciatus* (Reisen *et al.* 2006c). No previous study has detected WNV in field collected males of the five species positive in the current study. This is the first reported field evidence of vertical transmission of WNV in *Cx. salinarius, Cx. restuans, Oc. triseriatus, Ps. howardii* and *An. crucians*.

In the current study, WNV RNA was detected in a single pool of *Cx. restuans* collected in early May 2003. This succeeded the peak level of WNV infection in female *Cx. restuans* by several weeks. In a concurrent study, evidence of WNV was detected in 11 pools containing female *Cx. restuans* collected in March and April 2003 (Chapter 2, Table 2.3 and Table 2.5). In southern Louisiana, this species is abundant during the cooler months of the year, but adults are rarely collected from May through September (Chapman and Johnson 1985). In laboratory studies with *Cx. restuans*, a significant level of vertical transmission of a closely related flavivirus, SLEV, has been demonstrated. Nayar *et al.* (1986) reported a minimum infection rate

of 1:462 in the progeny of female *Cx. restuans* parenterally infected with SLEV. *Culex restuans* also is an efficient vector of WNV in the laboratory (Sardelis *et al.* 2001). Vertical transmission may enhance the overwintering and early season enzootic amplification of WNV in EBRP by *Cx. restuans*.

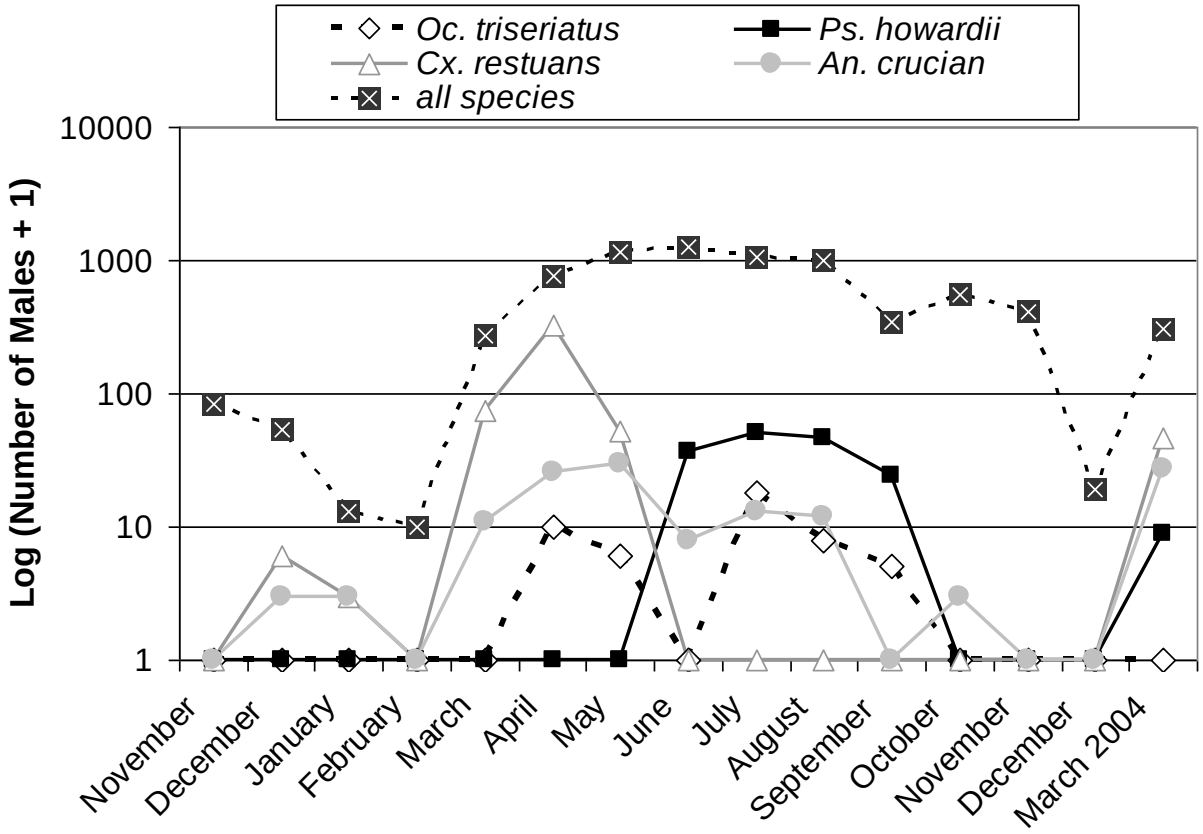

Figure 4. 1. Number of male *Oc. triseriatus, Ps. howardii, Cx. restuans* and *An. crucians* collected per month from sites in and adjacent to EBRP, from November 2002 to December 2003, and March 2004, and submitted for testing for WNV RNA by RT-PCR.

Infectious WNV was isolated from a single pool containing male *Cx. salinarius* collected from EBRP in late April, 2003. Vertical transmission of SLEV has been demonstrated in female *Cx. salinarius* infected by intrathoracic injection, but no virus was detected in the progeny of females infected orally by feeding on an infected host (Nayar *et al.* 1986). This species has

demonstrated to be a very highly competent vector of WNV in the laboratory (Sardelis et al. 2001), feeds primarily on mammalian hosts in EBRP (Chapter 3, Table 3.5), and is potentially an important bridge vector (Turell *et al.* 2005). Vertical transmission could greatly enhance the potential of this species to serve as a bridge vector by reducing the need for prior contact with an infective avian host.

Both WNV RNA and infectious WNV were detected in single pools containing male *Oc. triseriatus* collected from EBRP. This species is a competent vector of WNV in the laboratory, and may serve as a moderately important bridge vector (Turell *et al.* 2005). This species also is considered the primary vector of a California group virus, LACV, in many parts of the United States (Watts *et al.* 1972). Vertical and venereal transmission of LaCrosse virus (LACV) in *Oc. triseriatus* are important overwintering mechanisms for the virus, and may significantly contribute to amplification of the virus in the spring and summer (Watts *et al.* 1975, Thompson and Beaty 1978). It has been suggested that vertical transmission of flaviviruses occurs more readily in *Aedes* and *Ochlerotatus* species (Rosen 1987b). This is supported by laboratory experiments demonstrating significantly higher rates of vertical transmission of WNV in *Ae. albopictus* and *Ae. aegypti*, than in *Cx. tritaeniorhynchus* (Baqar *et al.* 1993). Like most *Aedes* and *Ochlerotatus* species implicated as vectors of WNV, the role of *Oc. triseriatus* is thought to be primarily limited to epidemic transmission (Turell *et al.* 2005), and possibly secondary enzootic transmission pathways among mammalian reservoir hosts (Tiawsirisup *et al.* 2005, Erickson *et al.* 2006). The current study suggests that *Oc. triseriatus* also could play a role in the overwintering maintenance of the virus.

In the current study, WNV RNA was detected in a pool containing male *An. crucians*, and a pool containing male *Ps. howardii*. Both pools were collected in the early spring.

Anopheles crucians can be very abundant in EBRP, with peak adult numbers usually occurring in the spring and fall months (Chapman and Johnson 1985). Vertical transmission could be partially responsible for the frequent WNV infections observed in pools containing females of these two mosquito species (Chapter 2, Table 2.2 to Table 2.4), as they rarely feed on avian reservoir hosts of WNV (Schaefer and Steelman 1969, Edman 1971, Apperson *et al.* 2004). Vector competence for WNV has not been evaluated in *An. crucians* or *Ps. howardii*. Although *An. crucians* can be an important pest in Louisiana (Chapman and Johnson 1985), neither *An. crucians* nor *Ps. howardii* are considered significant vectors of any arbovirus. However, *Ps. howardii* could represent a long term maintenance host for WNV. Unlike the eggs of *Culex* mosquitoes which immediately begin to embryonate and hatch within a few days after oviposition, *Ps. howardii* eggs can remain viable in the soil for several years before hatching (Breeland and Pickard 1967).

In the current study, males in three of the four pools positive for WNV RNA were collected in the spring. Although most of the male *An. crucians* and *Ps. howardii* were collected during the summer months when peak WNV infection rates in female mosquitoes are typically observed in EBRP (Gleiser *et al.* 2007), WNV RNA was not detected in males of either species collected during this time period. The two male pools positive for WNV by virus isolation also were collected in the spring, although the majority of male pools tested by virus isolation were collected prior to July 2003. No virus was detected in male mosquitoes collected during the winter months. The lower threshold for WNV replication in mosquitoes is between 10 and 14ºC (Reisen *et al.* 2006a). The average air temperature in Baton Rouge was 11.6ºC in February, 16.1ºC in March, 19.6ºC in April, and ≥ 25ºC from May through September, in 2003, and 18.1ºC in March 2004 (NCDC).

Temperature has a very strong influence on the developmental rate of the vectors and arboviruses, and the ability of the vectors to become infective. In *Cx. tarsalis* orally infected with WNV, extrinsic incubation temperature has a negative linear relationship with the length of the extrinsic incubation period, but a positive linear relationship with the total virus titres in infected females and the amount of virus expectorated during feeding (Reisen *et al.* 2006a). However, this linear relationship may not be valid for flavivirus infection in some mosquito species when they acquire the virus vertically. Infection rates in the adult F1 progeny of SLEV infected female *Ae. epactius* Dyar and Knab were 25 times greater when the F1 larvae were reared at 18ºC than at 27ºC (Hardy *et al* 1980). Similarly, Francy *et al.* (1981) reported that for *Cx. pipiens*, the rate of vertical transmission of SLEV at 18ºC was higher than the rate of vertical transmission of SLEV at 25ºC, though the difference was not statistically significant. Nayar *et al.* (1986) found a significantly higher rate of vertical transmission of SLEV in *Oc. taeniorhynchus* (Wiedemann) when the F1 larvae were reared at 18ºC rather than 27ºC. However, larval rearing temperature did not affect filial SLEV infection rates in *Cx. quinquefasciatus, Cx. salinarius, Cx. nigripalpus* Theobald, *An. quadrimaculatus* Say, and *An. albimanus* Wiedemann (Nayar *et al.* 1986). For WNV, a significantly higher infection rate was observed in the F1 pupae of WNV infected *Cx. tritaeniorhynchus* when they were reared at 20ºC rather than 26ºC (Baqar *et al*. 1993). However, Dohm *et al.* (2002a) failed to detect a difference in filial WNV infection rates in *Cx. pipiens* reared at 18ºC and 26ºC. Similarly, rearing temperatures between 20 and 32ºC did not significantly influence filial WNV infection rates in the F1 progeny of *Ae. albopictus* infected with Kunjin virus (Tesh 1980), a subtype of WNV. Further studies will be required to determine the influence of extrinsic incubation temperature on vertical transmission of WNV in a greater number of mosquito species, particularly species in the genera *Aedes* and *Ochlerotatus*.

Venereal transmission of WNV by mosquitoes has been demonstrated experimentally (Reisen *et al.* 2006c). Although the male mosquito infections detected in the current study represented a potential source of infection for female mosquitoes, the significance of venereal transmission on the WNV infection rate of female mosquitoes in the field is not clear. Reisen *et al.* (2006c) observed venereal transfer of WNV in *Cx. tarsalis*, from parenterally infected males to uninfected females, but only a small proportion of females retained the infection for 3-5 days post-mating, and no virus was detected in the progeny. However, these were very small scale experiments; only 21 females were successfully force-mated to infected males. Larger studies will be required to determine if venereal transmission represents a dead-end pathway for WNV.

Although vertical transmission of WNV has been observed in *Cx. quinquefasciatus*, both experimentally and in natural field populations (Goddard *et al*. 2003, Reisen *et al.* 2006c), we were unable to detect WNV RNA in any of the 83 pools containing a total of almost 4000 males of this species. Similarly, we did not detect WNV in 37 pools containing a total of over 1200 male *Ae. vexans*. It is possible that the numbers of specimens tested were insufficient to detect WNV in males of these two mosquito species. However, large pool sizes also may have reduced the sensitivity of our detection assay. Almost two-thirds (64.4%) of the pools of male *Cx. quinquefasciatus*, and almost half (46.6%) of the pools of male *Ae. vexans*, contained greater than 50 specimens. The sensitivities of the virus isolation method and the RT-PCR assay to detect arboviruses are negatively associated with mosquito pool size (Ksiazek *et al.* 1985, Lanciotti *et al.* 2000, Johansen *et al.* 2002). The four male pools testing positive for WNV RNA in the current study had very high Ct values, suggesting that viral titres in vertically infected males may be very low. West Nile virus may have been detected in a larger proportion of male pools if smaller pool sizes had been used in the current study.

In the current study, we were able to demonstrate evidence of vertical transmission of WNV in five species of mosquitoes in EBRP in 2003, including two potential bridge vectors (*Cx. salinarius* and *Oc. triseriatus*) and a potential enzootic vector (*Cx. restuans*). Although no vertical transmission was detected during the winter months, the majority of pools positive for WNV contained males collected in the spring, suggesting that vertical transmission in several vector species may play a role in the early season enzootic amplification of WNV. West Nile virus was not detected in any of the pools containing males of *Cx. quinquefasciatus*, the primary enzootic vector of WNV in EBRP. Further studies will be required to define the seasonality of vertical transmission, and the contribution of vertical and venereal transmission on the overall WNV infection rates in important vector species in southern Louisiana.

SUMMARY AND CONCLUSIONS

Prior to 1999, the primary mosquito-borne zoonoses of public health importance in the United States and Canada were LACV, SLEV, WEEV and EEEV. From 2002 to 2006, over 2500 human cases of WNV were reported each year in the United States alone. Arbovirus epidemics of this scale haven't occurred in the United States since the SLEV outbreaks in the 1970's (Zweighaft *et al.* 1979). In recent years, the impact of WNV on human and equine health and the associated costs of surveillance and vector control have been far greater than the combined impacts and costs associated with all other mosquito-borne pathogens of public health importance in the United States and Canada.

West Nile virus was detected for the first time in the Western Hemisphere during the summer of 1999. Within a few years, this invasive pathogen had successfully become established in a staggering diversity of zoogeographic regions within the hemisphere, from the temperate prairies in central Canada to tropical islands in the Caribbean. Although similarities may be drawn with other mosquito-borne arboviral agents endemic to North America, particularly SLEV, certain aspects of the epizootiology of WNV in North America are unique. No other mosquito-borne arbovirus of public health importance in North America naturally infects as wide a range of both invertebrate and vertebrate hosts. The geographical range of WNV in North America also is much greater than the geographical ranges of LACV, SLEV, WEEV or EEEV. As the range of WNV expands, the virus must adapt to the local environment, vertebrate hosts and arthropod vectors. The development of effective surveillance and control strategies for WNV requires a clear understanding of the epizootiology of the virus at a local or regional scale.

Between November 2002 and October 2004, 244,374 female mosquitoes of 36 species were collected is EBRP and tested for the presence of WNV. An additional 131,896 female

mosquitoes from EBRP were tested for WNV antigens in 2003, and 167,175 female mosquitoes from EBRP were tested for WNV RNA in 2004. These data represent the largest sampling effort undertaken to date for the purpose of identifying potential WNV vectors in Louisiana. Evidence of WNV infection was detected in specimens of 20 of the 37 mosquito species tested. The greatest number of WNV positive pools of mosquitoes contained females of *Cx. quinquefasciatus*, a species implicated as the primary enzootic vector of WNV and SLEV in Louisiana (Godsey *et al.* 2005b, Palmisano *et al.* 2005, Gleiser *et al.* 2007). Infectious WNV, and WNV antigens or WNV RNA, also were detected in pools containing specimens of *Cx. restuans*, *Cx. salinarius*, *Cx. nigripalpus*, *Ae. albopictus* and *Oc. triseriatus*; five species that have been implicated as important WNV vectors in the United States (Turell *et al.* 2005).

Consistent with a previous study in the parish (Gleiser *et al.* 2007), the results presented in this dissertation implicate the Southern House mosquito as the primary enzootic vector of WNV in EBRP. The dominant hosts of *Cx. quinquefasciatus* included several passerine bird species that have been implicated as competent WNV amplifying hosts. Transmission of WNV between *Cx. quinquefasciatus* and its two most common avian hosts, the Northern Cardinal and Northern Mockingbird, is likely responsible for a large proportion of the maintenance and epizootic amplification of WNV during the spring, summer and fall. Enzootic maintenance of WNV during the cooler months of the year in EBRP may be assisted by frequent contact between *Cx. quinquefasciatus* and two very competent avian reservoirs of WNV, the Blue Jay and the Common Grackle (Komar *et al.* 2003a). Contact between *Cx. nigripalpus* and the Northern Cardinal and the Common Grackle could represent a secondary enzootic cycle for WNV during the fall. Seasonal patterns of mosquito abundance and WNV infection observed in the current study also suggest that *Cx. restuans* is important as an enzootic vector in the early

spring. The WNV transmission cycle observed in this study is consistent with the pattern of enzootic WNV activity observed in St. Tammany Parish (Dietrich *et al.* 2005, Godsey *et al.* 2005b, Komar *et al.* 2005) and other locations in the eastern Gulf Coast region (Godsey *et al*. 2005a, Gibbs *et al.* 2006), but differs significantly from the pattern of transmission observed in other regions of the United States (Reisen *et al.* 2004b, Bell *et al.* 2006, Kilpatrick *et al.* 2006b, Cupp *et al.* 2007).

In addition to its role as an enzootic/epizootic vector of WNV, the data from this study is consistent with *Cx. quinquefasciatus* acting as the primary epizootic vector of WNV in EBRP. The temporal pattern of WNV infection rates in *Cx. quinquefasciatus* matched the temporal pattern of human infection in both 2003 and 2004. A significant proportion of bloodmeals were obtained by *Cx. quinquefasciatus* from human hosts in both years. In years when it is abundant in EBRP, *Cx. nigripalpus* also is potentially a significant bridge vector of WNV during the late summer and fall. Although no human bloodmeals were detected in *Cx. salinarius*, females of this species exhibited a very opportunistic feeding strategy, frequently using peridomestic mammals as hosts and were found to be infected with WNV in both 2003 and 2004, suggesting that there is a significant potential for *Cx. salinarius* to serve as a bridge vector.

For arboviruses with avian reservoirs, particularly WEEV, SLEV and WNV, it has been suggested that a seasonal increase in the proportion of the enzootic vector population feeding on mammalian hosts could be a major factor contributing to the shift from epizootic to epidemic transmission during the mid to late summer (Tempelis *et al.* 1965, Edman and Taylor 1968, Kilpatrick *et al.* 2006). Although a seasonal host shift in the feeding behavior of *Cx. quinquefasciatus* was not detected in the current study, it was found that *Cx. quinquefasciatus* fed on both humans and competent reservoir hosts thoughout the summer and fall when most

human infections are reported. A similar host feeding pattern was observed in *Cx. nigripalpus* during the fall months. These data suggest that, even in the absence of a seasonal shift in feeding behavior, *Cx. quinquefasciatus* and *Cx. nigripalpus* could serve as epidemic vectors of WNV in EBRP.

In the current study, males of at least 15 mosquito species were tested for the presence of WNV RNA. These data represent the largest number of species examined for evidence of WNV infection in field-collected male mosquitoes, and the first field evidence of vertical transmission of WNV in *Cx. salinarius, Cx. restuans*, *Oc. triseriatus, Ps. howardii* and *An. crucians*. Vertical transmission of WNV in populations of *Cx. restuans* and other mosquito species could be an important alternative maintenance strategy for WNV in EBRP during periods when horizontal transmission is reduced, such as overwintering. Evidence of vertical transmission in *Oc. triseriatus* and *Ps. howardii* introduce the possibility of long-term maintenance of WNV in quiescent eggs. Further studies should examine the role of vertical and venereal transmission on the maintenance and amplification of WNV in mosquito populations in EBRP and elsewhere.

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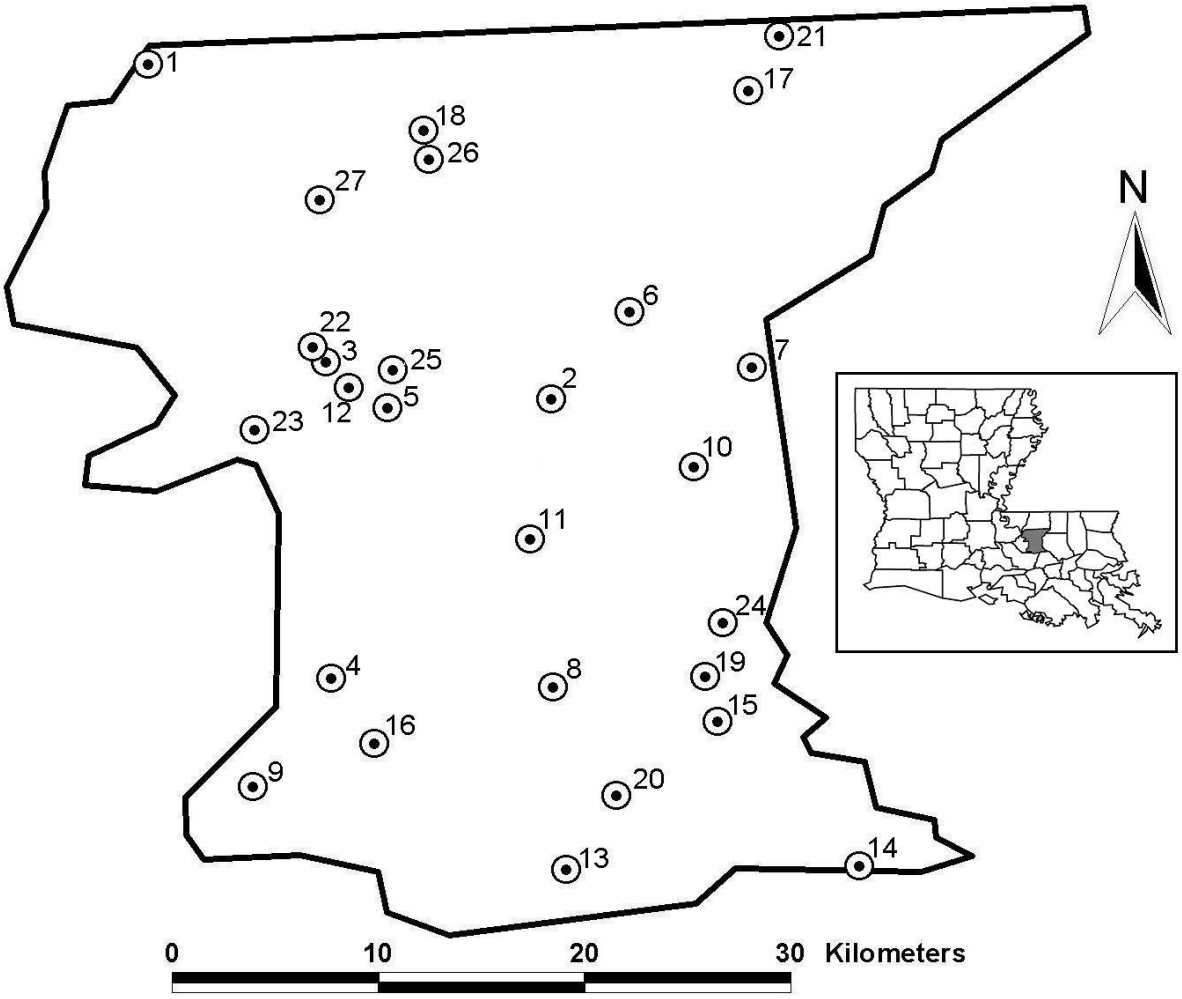
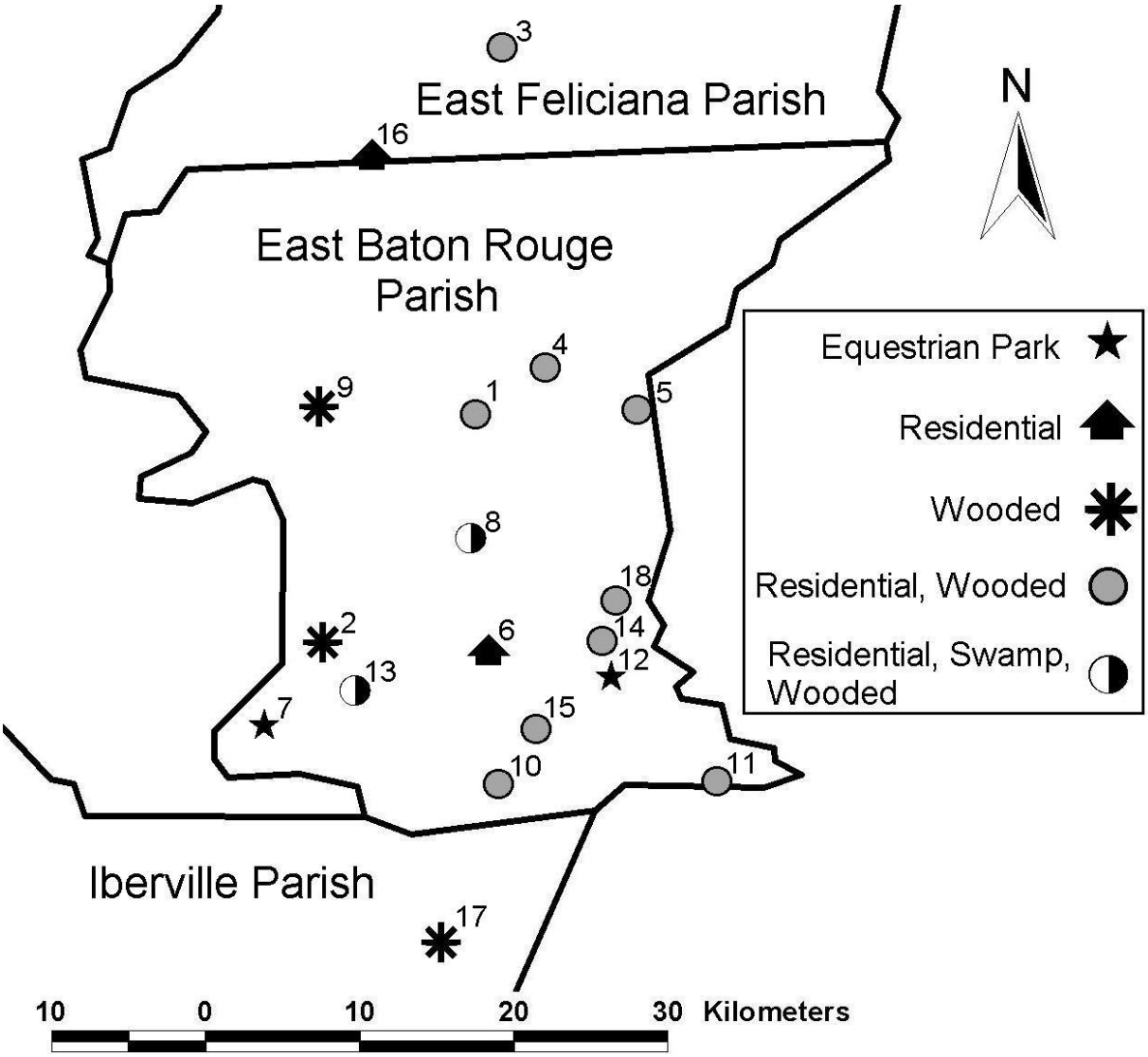
APPENDIX A: MAP OF BOARD OF REGENTS MOSQUITO SAMPLING SITES IN EAST BATON ROUGE PARISH



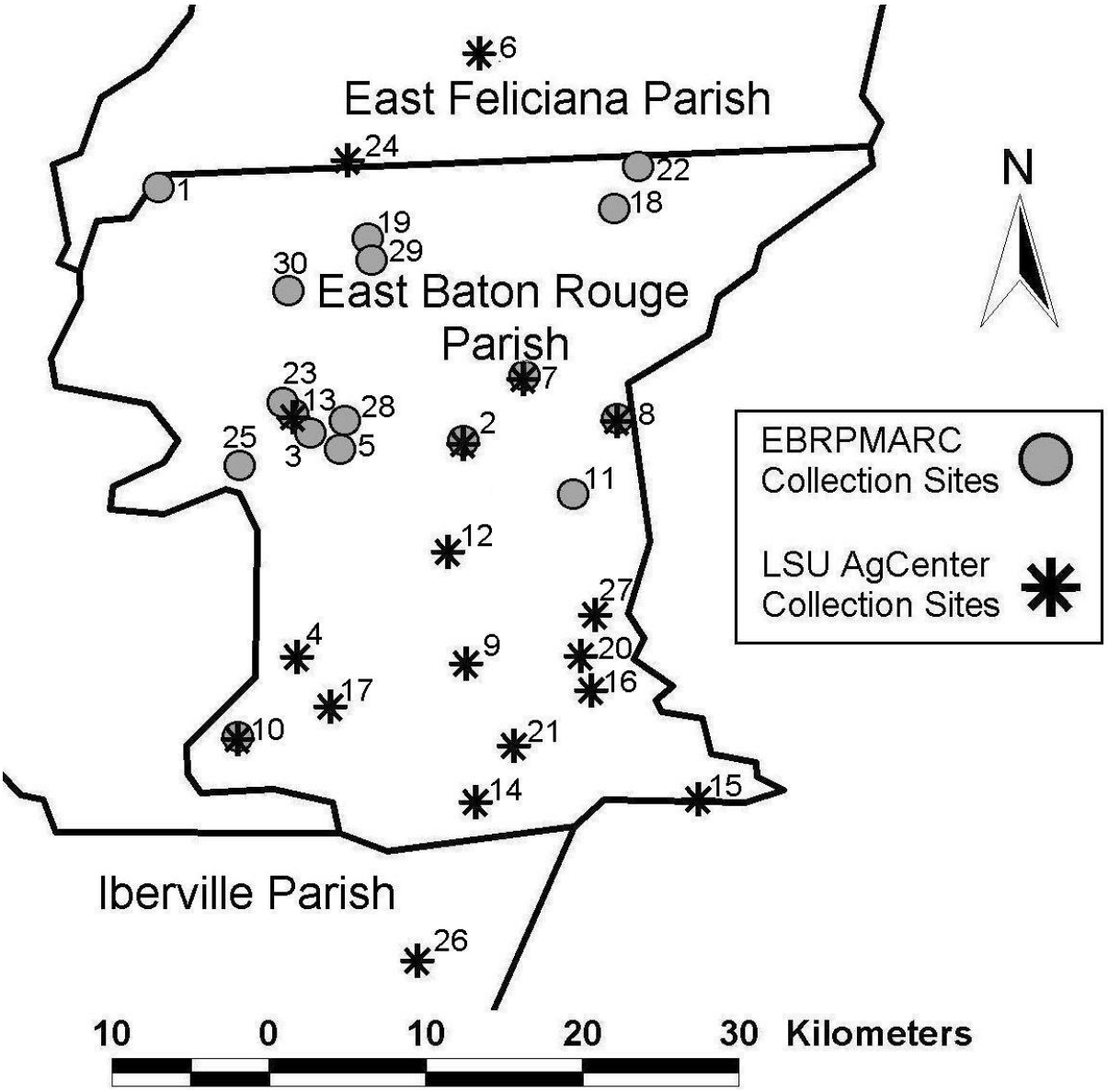
Key: 1=Bickham Rd., 2=Blackwater Rd., 3=Baton Rouge Zoo, 4=City Park, 5=Clark St., 6=Denham Rd., 7=Ednie Dr., 8=Emmet Bourgeois Rd., 9=Farr Park Horse Activity Center, 10=Greenwell Springs Park, 11=Greenwell Springs Rd., 12=Greenwood Park, 13=Highland Rd., 14=Hoo Shoo Too Rd., 15=Lazy B Stables, 16=Lee High School, 17=Liberty Rd., 18=McHost Rd., 19=Oneal Ln., 20=Pecue Ln., 21=Riley Rd., 22=South Magnolia Park, 23=Southern University, 24=Strain Rd., 25=Sunshine Rd., 26=White Bayou Park, 27=Willow Dr.

APPENDIX B: MAP OF SITES SAMPLED FOR BLOODFED MOSQUITOES IN EAST BATON ROUGE AND ADJACENT PARISHES



Key: 1=Blackwater Rd., 2=City Park, 3=Clinton, 4=Denham Rd., 5=Ednie Dr., 6=Emmet Bourgeois Rd., 7=Farr Park Horse Activity Center, 8=Greenwell Springs Rd., 9=Greenwood Park, 10=Highland Rd., 11=Hoo Shoo Too Rd., 12=Lazy B Stables, 13=Lee High School, 14=Oneal Ln., 15=Pecue Ln., 16=Slaughter, 17=St. Gabriel Research Station, 18=Strain Rd.

APPENDIX C: MAP OF SITES SAMPLED FOR MALE MOSQUITOES IN EAST BATON ROUGE AND ADJACENT PARISHES



Key: 1=Bickham Rd., 2=Blackwater Rd., 3=Baton Rouge Zoo, 4=City Park, 5=Clark St., 6=Clinton, 7=Denham Rd., 8=Ednie Dr., 9=Emmet Bourgeois Rd., 10=Farr Park Horse Activity Center, 11=Greenwell Springs Park, 12=Greenwell Springs Rd., 13=Greenwood Park, 14=Highland Rd., 15=Hoo Shoo Too Rd., 16=Lazy B Stables, 17=Lee High School, 18=Liberty Rd., 19=McHost Rd., 20=Oneal Ln., 21=Pecue Ln., 22=Riley Rd., 23=South Magnolia Park, 24=Slaughter, 25=Southern University, 26=St. Gabriel, 27=Strain Rd., 28=Sunshine Rd., 29=White Bayou Park, 30=Willow Dr.

APPENDIX D: PERMISSION LETTER

Andrew Mackay
Louisiana State University
Dept. of Entomology
402 Life Sciences Bldg.
Baton Rouge, LA 70803
13 November 2007

Sara Gazi
American Mosquito Control Association
15000 Commerce Parkway, Suite C
Mount Laurel, NJ 08054

Dear Sarah Gazi:

I am completing a doctoral dissertation at Louisiana State University entitled "Detection of West Nile virus activity in male and female mosquitoes, and evaluation of host-utilization patterns of mosquitoes, in East Baton Rouge Parish, Louisiana.". I would like your permission to reprint in my dissertation excerpts from the following:

Mackay, AJ, Roy, A, Yates, MM, Foil, LD. West Nile virus detection in mosquitoes in East Baton Rouge Parish, Louisiana, from November, 2002, to October, 2004. *J Am Mosq Control Assoc 24(1): in press.*

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If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope. Thank you very much.

Sincerely,

Andrew Mackay

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

Sarah Gazi

Date: 11/14/07

VITA

Andrew James Mackay was born to Jim and Margaret Mackay in Winnipeg, Manitoba, on May 20, 1968. He completed a Bachelor of Sciences in Agriculture at the University of Manitoba, majoring in Entomology, in 1992. In 1996, he completed a Master of Science; the title of his thesis was "The Influence of Temperature and Photoperiod on Ovarian Development in *Culex tarsalis* and *Culiseta inornata* (Diptera: Culicidae) in Southern Manitoba". Andrew briefly worked as a technician in the Department of Pediatrics and Child Health at the University of Manitoba, and at the Agriculture and Agrifoods Canada Cereal Research Center, before accepting a research associate position in the Department of Tropical Medicine at Tulane University. After leaving Tulane University, Andrew was employed as a research assistant by a Canadian company developing novel insecticides for the control of stored product pests. In 1999, he returned to Louisiana to work as a research associate for Dr. Lane Foil in the Department of Entomology at Louisiana State University. In the fall of 2002, Andrew began his dissertation research with Dr. Michael Perich. After Dr. Perich's untimely death in 2003, Andrew continued his graduate program under the direction of Dr. Lane Foil. Andrew's Doctor of Philosophy degree was confirmed in December 2007.