# IDENTIFICATION OF POTENTIAL MOSQUITO VECTORS OF WEST NILE VIRUS ON A FLORIDA ALLIGATOR FARM

By

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This document is dedicated to my husband, Dr. José Carlos V. Rodrigues, and to my father, Dr. Alfred J. Garrett, the two scientists who inspire me the most.

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Over the past several years, alligator farms in Florida, Georgia, and Louisiana have experienced sudden die-offs of juvenile and hatchling alligators (*Alligator mississippiensis*). These events occurred in the fall and tended to last two or three weeks. Histologic findings, virus culture, and RT-PCR evidence all suggest that the deaths were caused, at least in part, by infection with West Nile virus (WNV), a virus which is vectored by mosquitoes. Blood meal identification and virus screening were done in order to determine which mosquito species, if any, were involved in transmission of WNV on the farm. During September and October of 2003 four trips were made to an alligator farm in central Florida to collect mosquitoes inside and around the alligator pens. DNA was extracted from the abdomen of blood-fed individuals to test for the presence of alligator, avian, and mammal blood using PCR with different primer sets. Positives were confirmed with sequencing. The non-blood-fed mosquitoes were sorted into pools of up to 50 individuals and screened for WNV by inoculation onto Vero cells

and by RT-PCR with WNV-specific primers sets. A total of 4484 mosquitoes (sixteen different species and ten genera) were collected, 37 of which had visible blood meals. Three species (seven individuals) were positive for alligator DNA: *Culex erraticus*, *Mansonia dyari*, and *Mansonia titillans*. Other vertebrate blood meals were also identified: raccoon, horse, turkey, and pig from *Culex nigripalpus*, *Mansonia dyari*, *Culex nigripalpus*, and *Anopheles quadrimaculatus* and *Mansonia dyari* respectively. No virus was detected in any of the pools. This study was able to identify three mosquito species that fed on alligators, two of which (*Mansonia* spp.) have apparently not been recorded feeding on reptiles before. Studies on vector competence will be necessary to determine whether or not these mosquitoes are likely vectors of WNV on alligator farms.

# CHAPTER 1
# INTRODUCTION

West Nile Virus (WNV) is a Flavivirus (family Flaviviridae) and belongs to the Japanese encephalitis serogroup. It is an enveloped, positive sense single stranded RNA virus. WN virions are roughly spherical in shape and about 50 nm in diameter. WNV infects a large range of vertebrates as well as invertebrate vectors, most notably mosquitoes (Diptera: Culicidae) (Brinton, 2002).

West Nile virus was first isolated in 1937 in Uganda, from the blood of a woman suffering mild febrile illness (Smithburn et al., 1940, as cited by Hubalek and Halouzka, 1999), and records show that it was present and infecting humans, birds, and mosquitoes in Egypt in the 1950's (Melnick et al., 1951). Studies continued to expand the known range of the virus, and WNV (or evidence of its transmission) has now been found in many parts of Europe, the Middle East, Africa, China, and Southeast Asia. The closely related Kunjin (KUN) virus is present in Southeast Asia and Australia. With this large range, WNV is the most widespread flavivirus, although before 1999 it had not been reported in the Americas. It has been isolated from over 40 different species of mosquitoes in the Old World, with the genus *Culex* considered the primary enzootic and epidemic vector and several species of *Culex* and *Aedes* demonstrated as competent laboratory vectors. *Culex univittatus* Theobald is thought to be the principle vector in Africa and *Culex pipiens* Linnaeus in Europe (Hubalek and Halouzka, 1999). The virus is maintained in bird populations and spread with migrations (Rappole et al., 2000). Vertical transmission in mosquitoes has been detected and may contribute to maintenance

of the virus (Miller et al., 2000). In Europe, transmission to humans occurs during summer months (June to September) when mosquito vectors are most active (Hubalek and Halouzka, 1999).

Each year in South Africa, there were sporadic cases of WN viral disease (WNVD) often with mild illness. Two epidemics, one in 1974 and the other in 1984, marked a change in that normal activity. These epidemics may have been due to unusually high summer rains, which favored vector breeding and may have produced high vector population densities, which in turn promoted feeding on non-avian hosts, especially with the 1974 epidemic where more human cases were reported. Of all the WNV cases in South Africa, only four have involved more serious illness, and only one meningoencephalitis (Jupp, 2001).

In the late summer and fall of 1996, there was a major epidemic of WNVD in southeastern Romania with the highest clinical incidence in the urban center of Bucharest. WNV had been recorded in the area (by seroprevalence evidence) since the 1960's. This epidemic was the second largest recorded for Europe and was the first in which many clinical cases showed involvement of the central nervous system (CNS). Hospitals reported 17 deaths, and 400 cases of WN encephalitis, meningitis, or meningoencephalitis. Sampling following the epidemic showed that eight percent of the wild birds sampled and 41% of domestic birds had antibodies against WNV. Of about 6000 *Culex pipiens pipiens* L. aspirated from man-made structures around Bucharest, one was found positive for WNV, and the strain appeared to be most closely related to WNV strains from sub-Saharan Africa. Among the factors that may have facilitated this epidemic are the naivety of the population, the availability of flooded man-made

structures for mosquito breeding, and the summer drought that preceded the epidemic. In the years following the Romanian epidemic, cases (some fatal) continued to occur and seroconversions were observed in sentinel and domestic birds, although no WNV positive mosquitoes (out of 23,000 tested over two years) were found (Campbell et al., 2001).

After the 1996 Romania outbreak, other epidemics of WNV-induced CNS disease were reported in humans (including those in the United States, 1999-2004) (Lanciotti et al., 2002). The large Romanian epidemic would turn out to be only a part of an increasing trend of human and animal WNV outbreaks in Europe. Epidemics were reported in Italy in 1998 and in Russia in 1999 (Brinton, 2002). In late summer through fall of 2000, 131 WNV equine cases were reported in France, notably in an area with colonies of migratory birds and plentiful mosquito breeding habitat (Murgue et al., 2001) and during the fall of 2003 an outbreak caused disease in horses in Morocco (Schuffeneker et al., 2005). In 2000, an epidemic of WNV in Israel led to 326 hospitalizations and 33 deaths. Severe cases were mostly in the elderly and involved the CNS (Chowers et al., 2001). A study by Lanciotti et al. (2002) indicated that this increased severity of disease was likely due to the greater virulence of the lineage 1 virus responsible for these outbreaks.

In its Old World range, the virus appeared not to cause illness in wild birds with a few exceptions (Bin et al., 2001). Similar to birds in the Old World, reptiles and amphibians did not appear to suffer illness due to WNV, although evidence from multiple studies demonstrated that they were subject to infection. Seropositive turtles were found in Israel in the 1960's (Nir et al., 1969). Fourteen out of 20 healthy crocodiles (*Crocodylus niloticus*: five males and 15 females between 1 and 2.5 years old) at a farm

in the Negev Desert in southern Israel were found to be seropositive for WNV, though no deaths of crocodiles have been reported even during outbreaks of the virus in other animals (humans, horses, and geese) (Steinman et al., 2003). Frogs (*Rana* sp.) were also found with antibodies to WNV. Laboratory experiments showed that they could be infected by the bite of an infective mosquito and could later re-infect biting mosquitoes, thus demonstrating that they can be amplification hosts (Hubalek and Halouzka, 1999).

The first report of WNV in the Americas was from New York City in 1999. Since then the virus has spread north, south, west and has now been detected in all 48 states in the continental US except Washington (CDC, 2005), and has been reported in Canada (Buck et al., 2003), the Caribbean (Quirin et al., 2004), Mexico, and Central America, (Fernandez-Salas et al., 2003; Komar et al., 2003; Farfan-Ale et al., 2004; Cruz et al., 2005). The transmission cycle has paralleled that of the Old World: bird and mosquito (principally *Culex*) maintenance of the virus (Marfin et al., 2001; McLean et al., 2001) spread of the virus with migrating birds, and illness in humans and horses (Huang et al., 2002; Blackmore et al., 2003). The illness observed in humans and horses has been similar to that seen during the more recent European epidemics with the virus affecting the CNS in the more severe cases (Huang et al., 2002). Unlike in Africa and Europe, WNV in North America has caused the death of many different species of bird (McLean et al., 2001). Mortality in birds was so dependable that it actually became a warning system for WNV activity (Mostashari et al., 2003). This greater mortality could be due in part to the naivety of the birds in the New World, however, there is also experimental evidence showing that the strain of WNV isolated in New York in 1999 is more

pathogenic to crows than Old World strains from Australia and Kenya (Brault et al., 2004).

Sixty species of mosquito have been found infected with WNV thus far in the United States (CDC, http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm, 2005) and many of these are competent laboratory vectors of the virus. Specifically *Culex stigmatosoma* Dyar, *Cx. erythrothorax* Dyar, *Cx. nigripalpus*, *Cx. pipiens*, *Cx. quinquefasciatus* Say, *Cx. restuans* Theobald, *Cx. tarsalis* Coquillett, and *Cx. salinarius* Coquillett appear to be the most efficient enzootic vectors. Of these *Cx. tarsalis*, *Cx. salinarius*, and *Cx. erythrothorax* appear to have the greatest potential as bridge vectors although all have good potential. Other species like *Ochlerotatus triseriatus* (Say), *Oc. japonicus* Theobald, and *Aedes albopictus* Skuse have a potential to serve as bridge vectors (Turell et al., 2005). Not all species have been examined for their vector competence; no member of the Melanoconion subgenus of *Culex* has yet been evaluated (this subgenus is of special interest because some species are reptile-feeders). The impact of WNV on North American reptiles has not been examined as closely as that of birds and horses, and maybe there has been little impact overall. Common garter snakes (*Thamnophis sirtalis sirtalis* (Linnaeus)) and red-ear sliders (*Trachemys scripta elegans* (Wied-NeuWied)) did not develop detectable viremia after subcutaneous inoculation with WNV. North American bullfrogs (*Rana catesbeiana* Shaw) and Green iguanas (*Iguana iguana* (Linnaeus)) (infected by mosquito bite) did develop detectable viremia, although not more than $10^{3.2}$ PFU/mL (Plaque Forming Units, with one PFU equivalent to one viable virus particle) serum which is lower than needed to efficiently infect a biting mosquito such as *Cx quinquefasciatus* (Klenk and Komer, 2003; Jupp, 1974). Serious

morbidity was not noted (Klenk and Komer, 2003). In contrast, over the past several years, alligator farms in Florida, Georgia, and Louisiana have experienced sudden die-offs of juvenile and hatchling alligators (*Alligator mississippiensis* Daudin). These events occurred in the fall and tended to last two or three weeks. Histological findings, virus culture, and results from Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) all suggest that the deaths were caused, at least in part, by infection with WNV (Miller et al., 2003; Jacobson et al., 2005a).

## WNV in Farmed Alligators

In the US, alligators are grown commercially for their hide and meat, with the hide being the more valuable raw product. The value of a 2 m alligator (about three years old if grown in an intensive system) is about $US 150, with the major demand for hides and meat coming from Japan, Europe, and North America (Florida Fish and Wildlife Conservation Commission (FFWCC) report; Lane and King, 1989). About 1,500,000 crocodilian hides are traded per year with Florida, Texas, and Louisiana producing about 45,000 of that total, including hides from wild caught alligators. In 2003, Florida farms produced 22,527 alligators at a value of about $3.3 million (FFWCC report). Alligators are usually kept in temperature-controlled (ideally about 86° F, 30° C), dark pens and fed pellet feeds and raw meats (Lane and King, 1989).

The first group to describe the epizootics of WNV in alligator farms was Miller et al. (2003) when they investigated and reported on two die-offs occurring during the fall of 2001 and 2002 at a farm in southern Georgia. They observed “stargazing” before death, loss of leg control, and neck spasms in hatchling and juvenile alligators. Tissue was collected from the eye, thyroid gland, lymph node, lung, heart, brain, spinal cord,

kidney, liver, spleen, pancreas, adrenal gland, gallbladder, tonsil, trachea, stomach, intestines, and reproductive tract. Tissues and blood were subjected to RT-PCR, virus isolation, and bacterial culture. The appearance of the tissues and the RT-PCR results strongly suggested that West Nile virus was the cause of death, or had weakened the animals' immune systems such that bacterial infection set in. Raw horsemeat is a part of the alligator diet and was tested for WNV RNA. The meat that was fed to the alligators during the epizootics was positive for WNV by RT-PCR but was negative after the epizootic ended, leading the researchers to believe that virus in the horsemeat had caused the epizootic. Supporting this idea are experiments that have demonstrated that mice and hamsters can become infected when fed a fluid containing WNV (Odelola and Oduye, 1977; Sbrana et al., 2005). There have also been cases of predators becoming infected with WNV after eating infected prey (Garmendia et al., 2000; Austgen et al., 2004).

In Florida, similar epizootics occurred on several farms and one farm was investigated by Jacobson et al. (2005a). In 2002, an epizootic on a central Florida farm (named Farm A from here on) killed 300 of the 9000 alligators at the farm. Clinical signs in the alligators included anorexia, lethargy, tremors, swimming on the side, and opisthotonus. Tissues of three alligators were examined and showed signs of CNS disease and necrotizing hepatitis. Immunostaining revealed the presence of WNV antigen in multiple tissues. There was no evidence of two other pathogens that have previously been identified as disease agents in Crocodilians: *Mycoplasma* and *Chlamydia*. In contrast to the findings from Georgia, no secondary bacterial infection was apparent. Viremia in the infected alligators was greater than $10^{5.0}$ PFU/ml plasma making the alligators capable of infecting mosquitoes like *Cx. quinquefasciatus* and *Cx.*

*pipiens* (Jacobson et al., 2005a). Unlike the farm investigated in Georgia, Farm A feeds the alligators beef and alligator chow.

Illness occurred only in some pens on Farm A, with the affected pens containing multiple sick animals. Jacobson et al. (2005b) found that all blood sampled alligators that had shared a pen with sick animals during the epizootic carried WNV-neutralizing antibodies three months later, while those sampled from pens where no disease was recorded were not found to have such antibodies. This demonstrated that horizontal transmission had likely occurred inside the pens and suggested that the sporadic pattern of infection could be due to the infection of one alligator followed by viral shedding and infection of all of the other alligators sharing that pen. Laboratory experiments conducted by Klenk et al. (2004) confirmed the potential for horizontal transmission of WNV between alligators. In the laboratory, American alligators were injected subcutaneously with 7500 PFU of WNV or were fed viremic mice. All alligators developed viremia within three to six days. The viremia persisted for about ten days and reached approximately $10^6$ PFU/mL. Uninoculated tank mates also became viremic about a week after the inoculated alligators. Viral shedding from the cloacae was detected and was suspected to be responsible for horizontal transmission between tank mates. Two of the 29 infected alligators died, while the others developed WNV neutralizing antibodies within 25 days of the onset of viremia. These experiments demonstrated that horizontal transmission to tank mates does occur (100% in the study), viral shedding does occur, alligators can become infected through the oral route, and that the viremia of the alligators is high enough (Jupp, 1974) to infect biting mosquitoes making them potential amplification hosts of the virus.

Alligator farmers in Florida are not required to report the cause of death of their alligators, so there are no precise records of these epizootics, their impact, epidemiology, and timing. Florida farmers are required to report all deaths annually to the Florida Fish and Wildlife Conservation Commission, who then make this information available to the public. While it is impossible to make many conclusions based on gross annual records, it was clear in 2002 that some farms had virtually no unusual deaths while others apparently lost 10-50% of their alligators due to causes other than intentional slaughter (FFWCC 2002 annual report, and Dwayne Carbonneau personal communication).

## Mosquitoes as Vectors of WNV on Alligator Farms

There are two basic explanations for the source of the outbreaks of WNV on alligator farms, and they are not necessarily mutually exclusive. The first is that the virus is introduced by the bite of an infective mosquito, and the second is that the virus is introduced when the alligators are fed raw meat that contained active virus, an explanation supported by the findings of Miller et al. (2003). As of yet, no studies have been published that explore the potential for mosquito transmission of West Nile virus on alligator farms. The search for potential vectors of WNV in farmed alligators can be guided by a few criteria presented by Reeves (1957) (as reviewed in Turell et al. (2005)) and by Kilpatrick et al. (2005). A potential vector will repeatedly be found naturally infected with the virus and will be found in association (during the time when transmission is occurring) with the naturally infected vertebrate hosts, in this case, the alligators. If the potential vector is found in large numbers around the infected host, this should increase the chance that it is responsible for transmission (Kilpatrick et al., 2005). A potential vector should also be able to transmit the virus efficiently as demonstrated through laboratory competence studies. This study intended to identify potential

mosquito vectors of WNV in Florida farmed alligators by finding those mosquitoes that were numerous, and associated with (specifically feeding on) farmed alligators and determining if those associated mosquito species were also naturally infected with WNV.

**Blood Meal Identification**

A number of methods have been used to determine the hosts from which different mosquito species take blood meals. Observation of feeding mosquitoes, capture of mosquitoes in host baited traps, analysis of cytological characteristics of blood meals, analysis of serological characteristics of blood meals, and genetic information contained in blood cells have all been used to determine the host preferences of mosquitoes, with the last two of these five methods being the most commonly used today (Tempelis, 1975; Ngo and Kramer, 2003). The basic principle underlying the serological method is that antiserum (made when blood from various hosts is injected into other animals) will react with certain unidentified but unique elements in the blood of different hosts. Different techniques use this principle. In precipitin tests a suspension of the blood meal is mixed with antiserums against different vertebrates and if there is a reaction (portions of blood meal binding with antiserum) a precipitate forms and the meal is considered positive for that host type (Tempelis, 1975). The Enzyme-Linked ImmunoSorbent Assay (ELISA) test uses an enzyme-linked color change to signal when binding has occurred between the specific antibody and the reacting element in the blood meal. Fluorescent antibodies again rely on serology, with the fluorescence enhancing visualization of positive matches.

The technique developed most recently uses genetic characteristics of a blood meal to determine the host, in particular the technique relies on detection of specific regions of host DNA (usually mitochondrial) in the blood cells. Primers have been designed to amplify a region of the cytochrome b gene only for certain groups of vertebrates; there

are primer sets for all mammals, all birds, and for different orders of birds (Cicero and Johnson, 2001; Ngo and Kramer, 2003). Sequencing the fragment, followed by matching with known sequences in the BLAST database of GenBank, can confirm blood meal identifications or take the identification further, to family, genus, or species. By using these primers, host DNA could be detected in *Cx. pipiens* for up to 3 days after feeding (at 27°C) (Ngo and Kramer, 2003).

For these two techniques, naturally engorged females are collected from the field and the blood meal analysis is done in the laboratory. Different methods can be used to capture engorged females, and often the method chosen and the exact microhabitats sampled will depend on which mosquito species the study is targeting. The three collection methods used in this study were vacuum aspiration, CDC light traps (CDC = Centers for Disease Control and Prevention), and wooden resting boxes. With vacuum aspiration a battery-operated vacuum sucks mosquitoes against a screen until they can be transferred to a separate container. Aspiration can be done in vegetation, animal burrows, man-made objects/structures, and in natural and artificial crevices such as around tree roots or mosquito resting boxes. Vacuum aspiration has been used in Florida to collect *Ae. albopictus*, *Culex* of the subgenus Melanoconion, *Cx. nigripalpus*, *Culex*, *Aedes*, *Anopheles*, *Coquillettidia*, *Mansonia* and *Psorophora*. (Nieblyski et al., 1994; Edman, 1979; Day and Curtis, 1993; Edman, 1971).

A CDC light trap makes use of light and $CO_2$ to attract mosquitoes close to the trap where a fan-generated air current draws them into a collection jar or bag (Sudia and Chamberlain, 1988). In this study white incandescent lights were used. Field research in Florida and Georgia has shown white lights to be attractive to (among others)

*Uranotaenia sapphirina* (Osten Saken), *An. crucians* (Wiedemann), *Ae. vexans* (Meigen), *An. quadrimaculatus* Say, *Ae. atlanticus* Dyar and Knab, *Cx. nigripalpus,* and *Culex* of the subgenus Melanoconion (Love and Smith, 1957; Burkett et al., 1998). The addition of $CO_2$ as bait dramatically increases overall catch numbers of most mosquitoes (Burkett et al., 1998; Reisen et al., 1999). CDC traps are often left operating from before dusk until dawn in order to attract mosquitoes when their flight activity is maximum (Bidlingmayer, 1967).

Resting boxes are containers designed to resemble mosquitoes' natural resting places. They are often used to study host preferences because they attract females that are seeking a dark place to remain while digesting the blood meal and developing eggs. Resting boxes (with gray outside and red inside) set out on an island in the marshes near Vero Beach, Florida attracted Melanoconion *Culex* and *Uranotaenia* in swampy areas, and *Culiseta melanura* (Coquillett) and *Anopheles* near higher, hammock sites. Mosquitoes tended to enter during the mornings and leave during the day although some entered at all times (Edman et al., 1968).

A large body of work based on the different methods of host identification has allowed some generalizations about the feeding habits of different mosquito genera and species in North America. Species that fed exclusively on one class of vertebrate were perhaps the exception rather than the rule. Regional variation, seasonal variation, and habitat-linked variation in host preferences were observed. A number of different mosquito genera and species feed on reptiles and/or amphibians (ectotherms). Some appear to feed mostly on reptiles or amphibians, or even particular orders of ectotherms.

Others appear to take meals from reptiles only occasionally, while primarily feeding on mammals, birds, or both.

A number of studies from locations through out the eastern United States have found that some mosquitoes will occasionally take meals from reptiles. *Ae. atlanticus, Ochlerotatus. triseriatus,* and *Oc. sollicitans* (Walker) were found to feed on turtles, although in general mosquitoes of the genus *Aedes* fed on mammals and to a lesser extent birds (Tempelis, 1975). Turtle blood meals were identified from *Cx. salinarius, Cx. pipiens,* and from *Coquillettidia perturbans* (Walker) in New York. These three species were also found to feed on mammals and birds in the same locations (Appersen et al., 2002). In Florida, *Oc. infirmatus* Dyar and Knab, *Ae. taeniorhynchus* (Wiedemann), *Ae. albopictus, Ae. vexans, Culiseta melanura, Cx. territans, Cx. salinarius,* and *An. crucians* fed on one or more of the following reptiles: snake, turtle, and lizard (Edman, 1971; Edman et al., 1972**;** Nieblyski et al., 1994). In North Carolina, *Ae. atlanticus, Oc. henderson, Ae. vexans, Psorophora columbiae* (Dyar & Knab), *Ps. ferox* Humboldt, *Ps. howardii* Coquillett, *Cs. melanura, Cx. quinquefasciatus,* and *Cx. restuans* were all found with some reptile blood meals, though a majority of their meals were from non-reptilian hosts (Irby and Apperson, 1988). Seventeen percent of the meals identified from *Cs. melanura* in a Maryland study were from reptiles (Moussa et al., 1966). Of the engorged mosquitoes collected during a study in central Alabama, about 2% of *Cx. erraticus* Dyar and Knab were found to contain reptilian blood meals (Cupp et al., 2004). Animal baited traps in Delaware showed that *Oc. sollicitans, An. quadrimaculatus,* and *Cq. perturbans* occasionally fed on different reptiles but were better represented in traps with mammal or bird hosts (Murphey et al., 1967). Mosquitoes in the genus *Deinocerites* appear to be

opportunistic feeders, taking meals from mammals, birds, amphibians, and reptiles (Tempelis, 1975).

Many of these same studies also found species that took a majority or even all of their meals from ectotherms. The Delaware (Murphey et al., 1967) study found that *Cx. territans* Walker were frequently attracted to king snakes, water snakes, snapping turtles, and Eastern box turtles, but were not attracted to the mammals and birds tested. In Alabama Cupp et al. (2004) found that 75% of the *Cx. peccator* (Dyar & Knab) that they collected had fed on ectotherms, including one Crocodilian. In their North Carolina study, Irby and Apperson (1988) found that *Cx. territans* and *Cx. peccator* fed almost exclusively on reptiles and amphibians (about 99% of meals from ectotherms and 1% from birds). *Culex erraticus* and *Cx. territans* were collected from lizard (*Anolis carolinensis* Voigt*)* baited traps in north central Florida and readily fed on the lizards both in the traps and in the laboratory (Klein et al., 1987). *Ocholerotatus canadensis* Theobald (=*Aedes canadensis*) was the most frequent mosquito encountered around wild turtles in one study, and later research in North Carolina showed that 85% of the individuals sampled had taken their meal from an ectotherm (Irby and Apperson, 1988). With the wild turtles, most feeding took place around the head, neck, and legs, and sometimes between the scutes of the turtle's carapace (Crans and Rockel, 1968). These two studies also found that *Ae. triseriatus* was frequently attracted to or feeding on reptiles.

A study in Panama (Tempelis and Galindo, 1975) examined *Culex* species in the Neotropical subgenus Melanoconion (of which there are seven species in Florida) and found that four species fed mostly on lizards: *Cx. egcymon* Dyar (81%), *Cx. tecmarsis*

Dyar (89%), *Cx. elevator* Dyar and Knab (90%) and *Cx. dunni* Dyar (63%) while the other Melanoconion species in the study fed mostly on birds and mammals. This finding in Panama, that multiple *Culex* species in the subgenus Melanoconion feed on reptiles, is consistent with the findings in the United States.

Efforts to determine which species of mosquito(es) feed on alligators at a farm would likely have the greatest chance of success if they concentrated on sampling blood fed mosquitoes of the species that have already been identified feeding on reptiles. Based on previous Florida studies mentioned before, the three sampling techniques used (vacuum aspiration, CDC light traps, and resting boxes) should yield most, if not all, of the species that have been recorded feeding on reptiles, assuming that they occur in the vicinity of the farm.

In this study, the PCR-based method for analysis of blood meal was used, with Crocodilian-specific primers designed by Yau et al. (2002) that amplify a segment of chromosomal DNA and with Alligatoridae-specific primers based on work by Janke and Arnason, (1997), Ray and Densmore, (2002), and Glenn et al. (2002). The Alligatoridae-specific primers amplify a region of mitochondrial DNA, including portions of the cytochrome b gene, and genes for transfer RNAs. The location within the genome and the coding nature of the fragment amplified by the Crocodilian-specific primers are unknown.

A group of animals that contains enough viremic individuals to continually infect mosquitoes constitutes the reservoir, and the vertebrate reservoirs of WNV are most often birds (McLean et al., 2001). Thus a likely vector on the alligator farm would be a mosquito species that fed on both birds and alligators, such that it could move virus from

populations of infected birds to the alligators. To determine if the mosquito species found feeding on alligators were also feeding on birds, an avian-specific primer set was used. In addition, a mammalian-specific primer set was used to gain more information about the feeding habits of the mosquitoes captured around the alligator farm. These primer sets amplify a region of cytochrome b gene in the mitochondrial DNA for birds and mammals respectively (Ngo and Kramer, 2003).

**Screening Mosquitoes for WNV**

Potential vectors not only must feed on the host, but must also be infective. Mosquitoes collected from the alligator farm were tested for the presence of WNV. Work that is testing for viremic animals or for infected mosquitoes requires direct evidence of the virus particles (as opposed to testing for virus-neutralizing antibodies). Active virus from mosquito pools or tissues of viremic animals can be isolated in cell culture or the presence of viral nucleic acid can be demonstrated with strain-specific oligonucleotide primers and RT-PCR. In most recently published WNV research or surveillance reports, two tests (some using two different techniques) were often done to confirm a positive (and sometimes negatives as well). Often results from a real-time or standard RT-PCR test were confirmed with a second RT-PCR with a different primer set or with isolation of virus from the sample using cell culture (Kauffman et al., 2003; Lanciotti et al., 2000; Bernard et al., 2001). In this study, mosquito pools were tested for presence of virus by inoculation onto Vero cell monolayers, and by RT-PCR analysis with WNV-specific primers.

Kidney cells from the African Green monkey (Vero cells) are used in WNV isolation because they show cytopathic effects when infected by the virus, usually visible after three days (Odelola and Fabiyi, 1977). The virus binds to cells and enters by

receptor-mediated endocytosis (Chu et al., 2005). The capsid releases the positive single stranded RNA which is treated as messenger RNA by the cells and the single ~10,000 base pair open reading frame is translated into a single protein which is then cleaved by cellular and viral proteases (Brinton, 2002). Translation of the viral proteins is associated with the rough endoplasmic reticulum (Lee and Ng, 2004). The seven resultant non-structural proteins can then make a negative strand copy of the viral RNA, which serves as a template for new positive strand RNAs that can associate with structural proteins to form new virions. New virions move to the cell's margin in membrane vesicles, and are released by budding, individually at first and later in "bags" (Brinton, 2002). Budding of new virions starts within 10-12 hours after infection and is at maximum about 24 hours after infection (Ng et al., 2001). This process may perceivably slow the growth and division of the Vero cells, however, distinct cytopathic effects are usually first visible three days post-inoculation (Odelola and Fabiyi, 1977). Cells appear more rounded, with thicker, more distinct margins. They may appear "grainy" with vacuoles. As the cells die, they disconnect from the substrate. Vero cells are usually monitored for seven days after inoculation with mosquito homogenate (Kauffman et al., 2003). With virus isolation in cell culture, only active virus can be detected, and some work has suggested that it may not be as sensitive as RT-PCR (Nasci et al., 2002).

RT-PCR with primers specific for WNV was used to detect viral RNA in the samples. Two primer sets were used. Set one was used to screen the pools for WNV RNA and the second was used to confirm any positive bands from the first set. The first set, WN9483 and WN9794, were based on suggestions made by the CDC (based on work by Lanciotti). These primers amplify a 311 base-pair region within the NS5 gene, the

gene which codes for the viral RNA-dependent RNA polymerase (Lanciotti et al., 1999). This polymerase is the most highly conserved protein of West Nile virus (and of flaviviruses in general) (Brinton, 2002). As a consequence of the conserved nature of the region, WN9483 and WN9794 should readily bind to any potential strain of WNV.

The second set of primers, WN212 and WN1229, is based on suggestions of the CDC and work by Lanciotti et al., (2002). WN212 binds to a region within the gene for the viral nucleocapsid protein and WN1229 binds to a region within the envelope glycoprotein gene. The envelope protein gene is the more variable region of the WNV genome, but little genetic variation has been observed among US strains of WNV up to 2003 (Ebel et al., 2004), and new strains isolated since 2002 still have around 99.7% homology to strains isolated in New York in 1999 (Davis et al., 2004). Consequently this primer set will also likely bind to all potential strains of WNV.

The sensitivity of these approaches should allow for detection of mosquitoes that are potentially infective. To vector an arbovirus, a mosquito must have a minimum of about $10^5$ virions disseminated within its body (Hardy et al., 1983), and a fully disseminated infection in a mosquito with WNV is often more than this, about $10^{6.5}$ virions in the whole mosquito when measured 14 days after oral inoculation (Johnson et al., 2003). Mosquitoes encountered in the field may have lower titers than the minimum $10^5$ virions, titers that may be below the detection limit of the techniques applied in this study. However, because mosquitoes with such low titers are unlikely to be capable of efficiently transmitting WNV (Hardy et al., 1983), they are relevant to the search for potential vectors.

In general, the numbers of mosquitoes in a field collection that are found positive for WNV are low (Bernard et al., 2001), and it appears that the number of positives out of the total number collected (the Minimum Infection Rate = MIR) is not greater than 1 in a 1000 unless the collection was made in the vicinity of transmission (as demonstrated by human, horse, or bird cases) (Bernard et al., 2001.). However, it is difficult to make a generalization. MIR's vary considerably between studies and surveillance reports, and are likely influenced by the time of collection, the proportion of the collection comprised by "high risk" species like *Culex*, the age composition of the collections, and other factors that are difficult to quantify.

The objectives in this study were to identify potential vectors of WNV on Farm A using three of the four criteria described above. Mosquitoes were captured around the farm to determine which species fit the following criteria:

1. Species is present around host (alligators) during the time of transmission
2. Species is feeding on host
3. Species is infected with WNV

The fourth criteria, vector competence, was not addressed in this study.

# CHAPTER 2
# METHODS AND MATERIALS

Mosquitoes were captured, identified, and counted to determine which species were common around the farm. Mosquito blood meals were tested for presence of alligator DNA to determine which species were feeding on the alligators and were tested for the presence of avian and mammalian DNA to see if the alligator-feeding species were also feeding on other animals around the farm. Unengorged mosquitoes were screened for WNV to determine if any species had a high MIR.

## Mosquito Collecting

Four over night collecting trips were made to Farm A.

**Trip 1.** On September 9, 2003, the first collecting trip was made to Farm A alligator farm in Christmas, FL (Orange County, east of Orlando, on highway 50). At the time of this trip there had been multiple alligator deaths, many consistent with WNV infection. Equipment included one battery-powered backpack aspirator, plastic bags for collecting samples of feed and aspirator samples, a cooler with ice to keep samples cold during transit, and aerial nets for sweep net collecting in the vegetation around the farm.

Active collecting began mid-morning. The interior and exterior walls of pens and other buildings were visually scanned for resting mosquitoes. Insects were aspirated from vegetation, buildings and construction debris. Insects were also collected from vegetation using sweep nets. Around mid-day four CDC light traps (Sudia and Chamberlain, 1988) baited with $CO_2$ from dry ice were set. The dry ice was contained in an insulated plastic box with a small opening for outflow (MEDUSA Patent # 5,228,233

and # 5,272,179). Plastic tubing directed the flow of carbon dioxide from the metal box, through a bottle of water, and to the light trap. Small plastic vials with a sugar solution and a cotton stopper were taped inside of the collection jars of the CDC traps. Two traps were hung from low branches about 1 meter above the ground on trees along a chain-link fence that separated the farm from adjacent property (Fig 2-1).

Figure 2-1. CDC light trap set up on the western margin of the farm. $CO_2$ came from dry ice inside the insulated white plastic box.

The adjacent property was mostly wooded and was home to several pigs and at least one horse.

The other two CDC traps were hung inside of alligator pens where some alligator deaths had occurred in the past two years. They were hung from support pipes close to the door, also about 1 meter from the ground. The weather was sunny and warm when traps were set out and when collected.

Samples from sweep netting and aspirating were transferred to plastic bags, put on ice in the cooler, and taken to the lab in Gainesville where they were stored in -70°C until processed. The CDC traps were left over night. The traps were removed and samples collected the next day around the same time that they had been originally set up. Samples were put on ice, taken to Gainesville, transferred to plastic bags, and stored at -70°C until processed.

**Trip two.** A second collecting trip was made on September 24, 2003. Four CDC light traps were set inside four separate pens, each of which had housed alligators that died from illness consistent with WNV within the past two years. In addition, eight resting boxes (Moussa et al., 1966) were set up around the farm: four along the eastern margin of the farm, abutting a body of freshwater, three along the western margin of the farm close to the chain-link fence, and one inside of an alligator pen, where a CDC trap had also been placed. The resting boxes were wooden cubes roughly one foot on each side (30 cm) and open on one face. The open side of each box was fitted with a square of mesh and Velcro such that the mesh could be pulled down and secured over the opening to trap any mosquitoes that had gone inside the box. The outer surfaces of the box were

painted black with acrylic paint, and the inside surfaces were painted a maroon color (Fig. 2-2).

Figure 2-2. A 30 cm x 30 cm x 30 cm wooden resting box with black exterior and maroon interior was used to attract blood fed mosquitoes.

The CDC traps and resting boxes were set in the early afternoon on Sept. 25, left over night, and collected at about the same time the following day. There was light rain when the traps were set out and the weather was overcast with showers in the area when the traps were collected. The following steps were conducted to collect the mosquitoes from the resting boxes:

1. Boxes were approached from “behind” (the side opposite the open face);
2. From behind, screen was secured over the open face;
3. Boxes were then brought one at a time into the cab of a truck;
4. The screen was carefully pulled back and any mosquitoes aspirated with a Dustbuster vacuum fitted with a plastic tube. Any mosquitoes that escaped into the cab of the truck were also aspirated. Gauze was secured over the mouth of the

Dustbuster vacuum so that mosquitoes that were aspirated into the plastic tube would not be sucked into the Dustbuster.;

5. A gauze stopper was put in both ends of the plastic tube to trap mosquitoes, and tubes were then placed inside a cooler with ice.

**Trip three.** The third trip was made on October 17, 2003. Traps were set around 2:30 PM inside and outside of alligator pens, although records showing the exact locations of the traps were lost while in transit from Farm A to Gainesville. Traps were collected the following afternoon. Resting boxes were collected first, starting around 1:00 PM. All traps had been collected by 4:00 PM. All samples were kept on ice during the trip. For this trip and the following trip, the source of $CO_2$ bait was switched from dry ice to compressed gas in tanks. The regulators on the tanks were set to a flow rate of 500 mL/min. The weather was clear and warm both days.

**Trip four.** A fourth and final trip was made on October 24, 2003. Traps were set out around 3:00 PM. Two CDC traps were hung from trees on the eastern side of the farm, adjacent to the adult alligator lagoon. Three were hung inside of alligator pens: pen # 14 with small alligators and recorded deaths, pen # 15 with medium alligators and recorded deaths, and pen # 10 with medium alligators and no recorded deaths. One CDC trap was hung from the gate to the enclosure with the rectangular pens housing large alligators. One was hung from a tree in the middle of the farm and another was hung in the trees along the western margin of the farm adjacent to the neighboring property. Of the eight resting boxes, two were placed along the eastern edge of the farm adjacent to the lagoon, and the other six were set up along the western edge of the farm (Fig. 2-3). Traps were collected the following afternoon. The weather was clear and warm both days.

In Gainesville, mosquitoes were separated into pools of 1 – 50 individuals based on presence of blood meals, species (Darsie, 1998), trap type and number, and trap date.

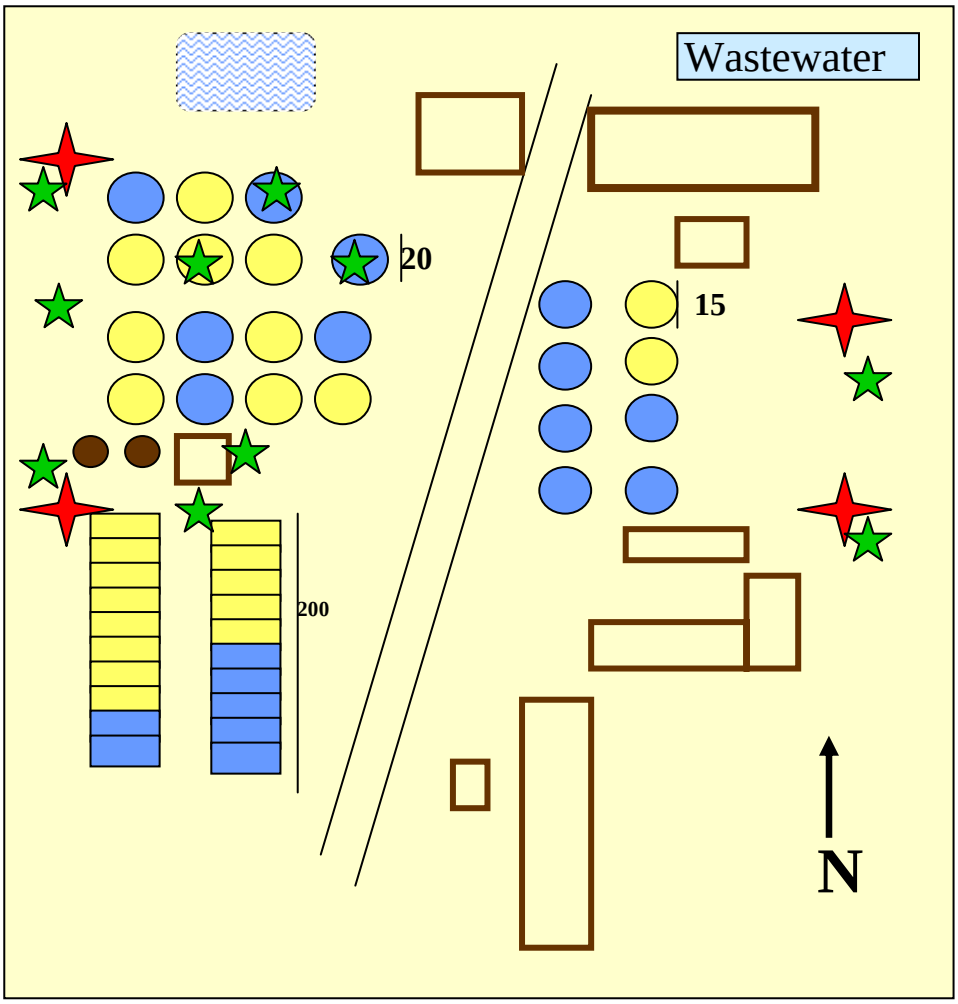


Figure 2-3. Map depicting layout of Farm A. Alligator pens where deaths had occurred are blue; pens with no history of deaths are yellow. Large red stars indicate where resting boxes were placed. Smaller green stars indicate where CDC light traps were placed. The structures indicated with brown outlines were buildings used for purposes like storage of maintenance equipment, housing for water heaters, basins for wastewater, and an office.

All identification and sorting was done on top of a chill table, and all pools were placed in tubes (blood fed mosquitoes singly in 1.5 mL microcentrifuge tubes, and non blood fed pools in 2 mL graduated microcentrifuge tubes (OPS, Petaluma, CA) with 1-2 copper-clad steel beads (BB-caliber airgun shot)) and stored at –70°C until processed.

Each pool was assigned a code name. For the pools of unengorged mosquitoes, the code names started with a digit one through four that corresponded to the collecting trip when mosquitoes were captured. Letters were used to designate each pool, and the code ended with a digit that indicated the trap the mosquitoes were from. For the engorged mosquitoes the first digit also designated the collection trip and the letters were shorthand for the genus and/or species of mosquito, BF stood for "blood fed", and the end digits described either the trap number or were used to distinguish multiple mosquitoes that were from the same species, date, and trap number.

**Blood Meal Identification**

Prior to working with field-collected mosquitoes, extraction and PCR procedures were tested and optimized on positive controls. To form a positive control for the alligator bloodmeal study, *Ae. aegypti* Linneaus and *Cx. quinquefasciatus* mosquitoes were obtained from the USDA (United States Department of Agriculture), Gainesville colonies. These mosquitoes were starved for 24 hours and then offered one of two liquids using the membrane feeding system (Davis et al., 1983; McKenzie, 2003). Briefly, one mL of the liquid was placed into the depression in the bottom of a film canister lid, a square of membrane (bridal veil with a layer of silicon) was placed so that it covered the depression, and then the membrane was secured over the canister lid using a plastic ring. This membrane feeding system was then inverted and put on the top the wire-mesh mosquito cages such that mosquitoes could insert their proboscis through the mesh of their cages, through the silicon layer of the membrane, and into the liquid. The two liquids offered to the mosquitoes in this manner were: heparinized alligator blood and meat juice from previously frozen alligator tail meat that was sweetened with 10% sucrose sugar (Figure 2-4). The sugar was added to encourage feeding (Aissa

Doumbouya, personal communication). The alligator blood was drawn from the sinus vein of an alligator patient at the large animal clinic of the University of Florida School of Veterinary medicine, and was provided by Dr. Darryl Heard (use of blood approved, UF animal use protocol #D687). Mosquitoes were allowed to feed for 24 hours after which they were frozen at –20°C, and the engorged individuals were separated for later use.

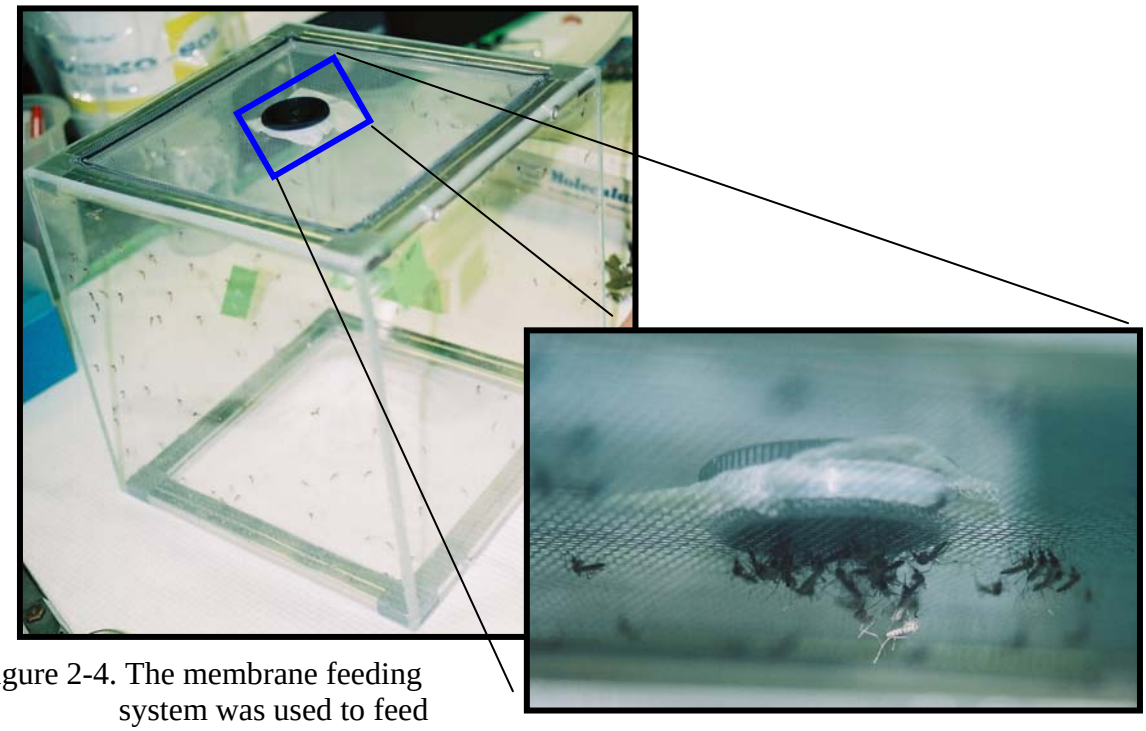

Figure 2-4. The membrane feeding system was used to feed alligator blood and alligator meat juice to *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes to be used as positive controls when testing field-collected mosquitoes for the presence of alligator blood.

The positive controls for testing the avian-specific and mammal-specific primers were a mosquito fed on a live chicken (feeding that is a normal part of colony maintenance at the USDA) and a *Coquillitidia perturbans* captured after it had fed on the investigator. After it was established that the avian primers worked for the chicken-fed

mosquito, DNA from a rock dove (*Columba livia* G.F. Gmelin) was used as the avian positive control for PCR reactions.

The abdomens of both the engorged positive control mosquitoes and an un-engorged negative control mosquito were removed, placed separately into 1.5 mL plastic tubes, and homogenized in 250 µl of buffer 1 (buffer 1 contains 0.32 M sucrose, 50 mM Tris at pH 7.25, 10 mM $MgCl_2$, and 0.5% NP 40 detergent) using a plastic mortar. The tubes were then centrifuged at 6000 rpm for four minutes to pellet the mosquito cells and parts, and the supernatant was discarded. The pellet was resuspended in a second buffer (75 mM NaCl, 25 mM EDTA, and 10 mM Tris at pH 7.8) to lyse the cells. Fifteen µl of 0.5 M EDTA, 15 µl of 20% SDS, and 8 µl of proteinase K (20 mg/mL) were added and the mixture was incubated overnight in a 55 $^{o}$C water bath. The following day the tubes were centrifuged at 13,000 rpm for 10 minutes and the supernatant was transferred to a new tube. Twenty µl of RNAse (5 mg/ml) was added and the mixture was incubated at 37 $^{o}$C for one hour. Following incubation, DNA was separated using phenol and chloroform followed by a second precipitation using only chloroform. DNA was precipitated from the aqueous phase with 600 µl of cold 95% ethanol followed by centrifugation (13,000 rpm at 4 $^{o}$C for 10 minutes). The ethanol was then removed and the pellet was vacuum dried. The DNA was resuspended in 30 µl of 10 mM Tris and stored at –20 $^{o}$C until used in PCR reactions. Three other DNA extraction protocols (TRIZOL®, C-TAB, and DNeasy) were tried on the positive controls. Only the one described above was used on the field samples because it was the easiest protocol that consistently gave good final DNA concentrations.

The DNA was tested to determine its vertebrate origin with multiple primer sets: Crocodilian-specific primers described by Yau et al. (2002), mammal-specific primers described in Ngo and Kramer (2002), and bird-specific primers described by Cicero and Johnson (2001). In addition primers for alligators were designed for this study based on the suggestions of Glen et al. (2002). Primers were made using the Primer3 program and the mitochondrial genome of *A. mississippiensis* from GenBank, accession number Y13113, (Janke and Arnason, 1997) (Table 2-1). This primer set will be referred to as the alligator-specific set, although they may also amplify DNA from other members of the Alligatoridae family or Crocodilian order. The Crocodilian-specific primers amplify a region of chromosomal DNA, while the alligator, avian, and mammalian primers amplify a mitochondrial region including parts of the cytochrome b gene. The conditions used with the mammal and bird primers closely followed those described in the original papers. For the Crocodilian and the alligator primers several optimization experiments were done to find the conditions under which the positive controls would consistently amplify. These experiments tested for optimal concentrations of $MgCl_2$, primers, template DNA, and for the optimal annealing temperature. Once positive controls were working consistently, DNA was extracted from the field-collected mosquitoes using the same protocol as before, and each mosquito sample was tested for vertebrate DNA using each of the four primer sets. Reaction conditions and the thermocycle program for each of the primer sets are described in Table 2-3.

The PCR products were run on 1% agarose gels stained with ethidium bromide. Any bands were excised from the gel, DNA was purified using the QIAquick Spin kit for gel extraction (Quiagen, Valencia CA) following the handbook protocol (Appendix A),

and the fragments were sequenced using the BigDye Terminator Cycle Sequencing kit (PE Biosystems, Foster City CA) following a protocol modified from the kit instructions (Appendix B). Sequences were run at the University of Florida ICBR (Interdisciplinary Center for Biotechnology Research) core facility in Gainesville, Florida. The sequences were then edited using Sequencher™ version 4.1 software (Gene Codes Co., Ann Arbor, MI) and compared to all those on the BLAST database (GenBank) to identify hosts with more certainty and specificity.

For the three samples that did not produce clear sequences, the PCR products were inserted into pGEM®-T vector (Promega, Madison, WI) according to the kit instructions (Appendix C) with an overnight incubation at 4 $^{o}$C. Following incubation the vectors were prepared for transformation into *Escherichia coli* bacteria by heat inactivating the ligase at 65 $^{o}$C for 10 min, diluting the DNA (x three) with sterile water, and sterilizing the DNA with 300 µl of ether. Vectors were then transformed into bacteria. Five µl of the DNA ligation mixture was mixed with 50 µl of competent bacterial cells, and the combination was incubated for 30 min on ice and then heat shocked for 30 s at 37 $^{o}$C. The cell mixture was left for 2 min on ice, then 0.95 mL of medium (deionized water with 2% bacto-tryptone, 0.5% bacto-yeast extract, and 0.05% NaCl) was added and the bacteria were set in a 37 $^{o}$C water bath and shaken at 225 rpm for 1 h. This mixture was then plated onto LB agar plates with 100 µg/mL ampicillin and 20 µg/mL X-gal, plates were incubated at 37 $^{o}$C overnight, transformed colonies were selected, and transformed bacteria were grown overnight in media (4 mL LB medium with 5 mg/mL ampicillin) at 55 $^{o}$C. The plasmid was removed using the QIAprep™ Spin Miniprep kit (QIAGEN, Valencia, CA) following kit instructions (Appendix D), and the insert was removed from

the plasmid by digestion with EcoRI at 37 $^{o}$C in a mixture containing 7 µl water, 1 µl reaction buffer, 0.3 µl EcoRI, and 2 µl plasmid DNA. The insert was then sequenced as before.

Table 2-1. Primers sets in PCR used to amplify DNA from different vertebrate hosts.

| Host | Primer | Sequence | Product size(bp) | Reference |
|---|---|---|---|---|
| Alligator | Forward | CGCTTCACTGCCCTACACTT | 850 | Current study |
| | Reverse | GCTTTAGTGTTTAAGCTACGATAACTG | | |
| Crocodilian | Forward | GATGTGGACCTTCAGGATGC | 209 | Yau *et al.* (2002) |
| | Reverse | CAGAGGTTCAATCCACGGTT | | |
| Avian | Forward | GACTGTGACAAAATCCCNTTCCA | 508 | Cicero and Johnson (2001) |
| | Reverse | GGTCTTCATCTYHGGYTTACAAGAC | | |
| Mammalian | Forward | CGAAGCTTGATATGAAAAACCATCGTTG | 772 | Ngo and Kramer (2003) |
| | Reverse | TGTAGTTRTCWGGGTCHTCTA | | |

**Virus Detection**

About 12 hours prior to virus work, each well of 24-cluster well plates was inoculated with 5.0 x $10^4$ Vero cells in 1 mL cell culture media (media: Lebovitz L-15 media, 10% fetal bovine serum, 100 U of penicillin/streptomycin, 100 µg/mL gentimicin, and 1 µg/mL amphotericin B (Fungizon)). Plates were kept over-night in a 37 $^{o}$C incubator and used for virus isolation the following day.

Mosquitoes were processed in a Biosafety Level 3 laboratory (BSL-3 lab). Pools were homogenized for 1 minute in 1 ml of diluent (Phosphate Buffered Saline (PBS, contents: 0.8% NaCl, 0.02% KCl, 0.144% $Na_2HPO_4$, and 0.024% $KH_2PO_4$ in distilled $H_2O$, pH of 7.4) with 4% Fetal Bovine Serum (FBS)) using a laboratory mixer.

Following homogenization tubes were centrifuged at 13,700 rpm for 10 minutes to pellet mosquito solids. The mosquito supernatant was removed to a new tube and 200 µl and 10 µl were removed for use in screening. Any remaining homogenate was frozen at –70 $^{o}$C until needed further.

For the RNA extraction, the 200 µl of homogenate was then added to a tube containing 600 µl of Trizol LS reagent (Life Technologies, Gaithersburg, MD), and the mixture was incubated for 5 minutes to inactivate the virus. After incubation, tubes were removed from the BSL-3, stored at –70 $^{o}$C, and later RNA was extracted as described in the Trizol manufacturer's instructions (Appendix E) and was resuspended in 30 µl of nuclease-free water. All RNA samples were then tested for WNV RNA using Promega Access RT-PCR System (Promega, Madison, WI) with the following concentrations of reagents: 5 pmol of each primer, 1X kit reaction buffer, 0.2 mM each dNTPs, 2 mM $MgSO_4$, 1 unit/reaction of both Taq polymerase and Reverse Transcriptase, and 1 µl of template for 25 µl of reaction mix (Table 2-3). The primers used were WN9483 and WN9794, (Table 2-2) and the thermocycle was run in a PTC-200 (Table 2-3). The RT-PCR products were visualized on 1% agarose gels with ethidium bromide staining. All bands were excised from the gel, cleaned, and sequenced as described for the vertebrate primers (see Appendix A and B). The samples showing positive bands with WN9483 and WN9794 were also confirmed using a second primer set, WN212 and WN1229 (Table 2).

Table 2-2. Primers sets used in RT-PCR to test for the presence of WNV RNA.

| | Primer* | Sequence | Amplicon size (bp) | Annealing region |
|---|---|---|---|---|
| Confirmation Set | WN212 | TTGTGTTGGCTCTCTTGGCGTTCTT | 1071 | Capsid protein |
| | WN1229 | GGGTCAGCACGTTTGTCATTG | | Envelop glycoprotein |
| Screening | WN9483 | CACCTACGCCCTAAACACTTTCACC | 311 | NS5: RNA-dependent RNA polymerase |
| Set | WN9794 | GGAACCTGCTGCCAATCATACCATC | | NS5: RNA-dependent RNA polymerase |

* WNV-specific primer sets

Ten μl of the mosquito supernatant was mixed with 100 μl of cell culture media to be used as an inoculum for the Vero cells. Media was removed from the prepared wells of the 24 cluster well plates and the inoculum was added. Inoculated plates were incubated for one hour at 37 $^{o}$C with gentle hand rocking every ten minutes. After incubation 500 μl of cell culture media was added (media: Lebovitz L-15 media, 10% fetal bovine serum, 100 U of penicillin/streptomycin, 100 μg/mL gentimicin, and 1 μg/mL amphotericin B (Fungizon)) to each well and plates were placed inside a plastic box with moistened paper towels and kept in a 37 $^{o}$C incubator. Cells were checked daily for 7 days for cytopathic effect (CPE) using an inverted compound microscope with WNV CPE expected to begin on days 3 or 4 post-inoculation.

Samples that showed signs of bacterial contamination were recorded as such, and the homogenate for that sample was thawed and the inoculation was repeated with a new well of Vero cells. In these cases, it was assumed that the homogenate was the source of the contamination and for the new well, the inoculum was removed after the one-hour

Table 2-3. Reagent concentrations and thermocycle conditions used for PCR with vertebrate-specific primer sets and RT-PCR with WNV-specific primer sets.

| Primer set | PCR reagents | Concentration | Thermocycle |
|---|---|---|---|
| Mammalian | Buffer | 1X | 93 for 3 min |
| | dNTP's | 0.2 mM each | 94 for 30 sec |
| | each primer | 5 pmol/rxn | 50 for 30 sec |
| | $MgCl_2$ | 4 mM | 72 for 1 min 30 sec |
| | Taq polymerase | 1 unit/rxn | Goto 2 45 times |
| | template DNA | 1 µl | 72 for 3 min |
| Avian | Buffer | 1X | 93 for 3 min |
| | dNTP's | 0.2 mM | 94 for 30 sec |
| | each primer | 15 pmol/rxn | 50 for 30 sec |
| | $MgCl_2$ | 2.0 mM | 72 for 1 min 30 sec |
| | Taq polymerase | 1 unit/rxn | Goto 2 34 times |
| | template DNA | 1 µl | 72 for 3 min |
| Crocodilian | Buffer | 1X | 94 for 3 min |
| | dNTP's | 0.2 mM | 94 for 30 sec |
| | each primer | 10 pmol/rxn | 53 for 30 sec |
| | $MgCl_2$ | 2.5 mM | 72 for 30 sec |
| | Taq polymerase | 1 unit/rxn | Goto 2 40 times |
| | template DNA | 2 µl | 10 for ever |
| Alligator | Buffer | 1X | 93 for 3 min |
| | dNTP's | 0.2 mM | 94 for 30 sec |
| | each primer | 10 pmol/rxn | 55 for 30 sec |
| | $MgCl_2$ | 2 mM | 72 for 1 min 30 sec |
| | Taq polymerase | 0.2 µl/rxn | Goto 2 34 times |
| | template DNA | 1 µl | 72 for 3 min |
| WNV | Buffer | 1X | 48 for 5 min |
| | dNTP's | 0.2 mM | 94 for 5 min |
| | each primer | 0.4 pmol/µl | 95 for 30 sec |
| | $MgSO_4$ | 2 mM | 58 for 45 sec |
| | Taq polymerase | 1 unit/rxn | 68 for 2 min |
| | Reverse transcriptase | 1 unit/rxn | Goto 3 39 times |
| | Template RNA | 1 µl | 68 for 10 min |

incubation period in an attempt to remove the contaminated homogenate after the inoculum had inoculated.

Assuming one infective mosquito in the pool contained $10^{6.5}$ virions, this method would produce an inoculum with about $10^{4.5}$ virions. This inoculum placed into a well with 5 x $10^4$ cells would yield a Multiplicity of Infection (MOI) of approximately 0.63.

Three positive controls were conducted simultaneously with samples. Each control used a previously frozen Florida isolate of West Nile virus, WNV-FL01-JC2-3C2P2, which had been at a titer of approximately $10^{7.5}$ $TCID_{50}$/mL before freezing. In one control well, 50 µl of the virus was added directly. For the other two controls, 2 and 100 µl of the virus were added to tubes each containing 38 *Ae. aegypti* colony mosquitoes, and these controls were then processed in the same manner as the field samples. For these two controls the inoculation contained approximately $10^{2.5}$ and $10^{4.5}$ virions respectively. This gave an MOI of about 0.0063 and 0.63 respectively. For the direct inoculation of 50 µl, the MOI was about 32.

# CHAPTER 3
# RESULTS

## Mosquito Collecting

During collecting no mosquitoes were seen resting inside of the alligator pens or on other buildings. Mosquitoes were observed in the brush and woods along the western margin of the farm. These swarmed if one entered the woods but did not attempt to feed on collectors in the open. In several instances, mosquitoes, *Mansonia* sp., pursued and bit the investigator in the open during daylight hours (1:00 to 3:00 PM). Inspection of the pens revealed that while they were mostly closed, there were cracks and spaces around the doors and pipes that would be sufficient for mosquitoes to enter and exit.

Collection bags on several of the CDC traps that were hung inside alligator pens were apparently torn down by the alligators some time during the night. These samples could not be recovered. Taking into account the losses due to alligator interference and one disturbed collection bag there was a total of 20 trap nights for the CDC light traps and 24 trap nights for the resting boxes over the four collecting trips.

A total of 4484 unfed and 37 blood fed mosquitoes was collected from CDC traps, resting boxes, and aspiration. There were 16 species (10 genera) represented in the collection. The species were *An. quadrimaculatus, An. crucians, Mansonia dyari, Ma. titillans, Cx.. nigripalpus, Cx. erraticus, Cx. quinquefasciatus, Uranotaenia sapphirina, Ur. lowii, Psorophora columbiae, Ps. ferox, Coquillettidia perturbans, Wyeomyia vanduzeei, Culiseta. melanura, Ae. albopictus,* and *Oc. infirmatus* (Fig 3-1). The numbers of mosquitoes collected varied from one trip to the next.

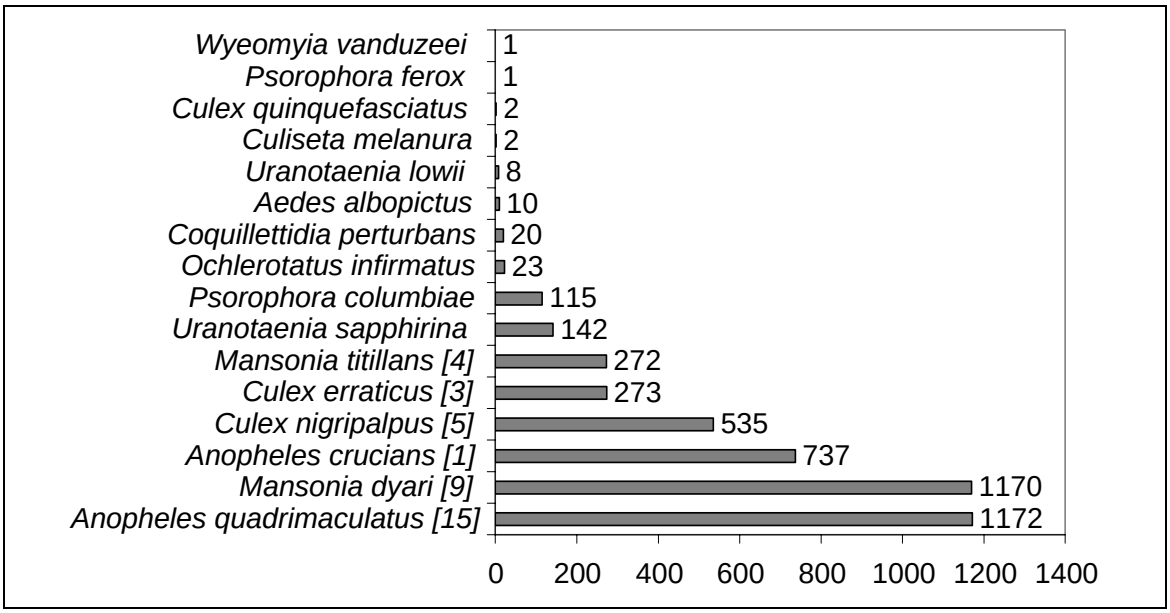


Figure 3-1. Total mosquito numbers collected over four trips to Farm A. Numbers in [ ] indicate engorged mosquitoes.

Five different species were captured in resting boxes and overall the percent of blood-fed individuals was greater in resting boxes than in CDC light trap collections (31.5% versus 0.4%). In the resting boxes there were *An. quadrimaculatus* (37 total, 15 blood-fed), *An. crucians* (6, 1), *Cx. erraticus* (4, 2), *Cx. nigripalpus* (9), and *Cx. quinquefasctiatus* (1). One blood-fed *Cx. nigripalpus* was captured with vacuum aspiration.

Seven different species were collected from CDC traps that were located inside of alligator pens. These seven species were *An. quadrimaculatus, An. crucians, Ma. dyari, Ma. titillans, Cx. nigripalpus, Cx. erraticus,* and *Cq. perturbans.* Based on average numbers of the different species in traps located inside and outside of the pens, it appeared that some species more readily entered pens than others (Fig. 3-2).

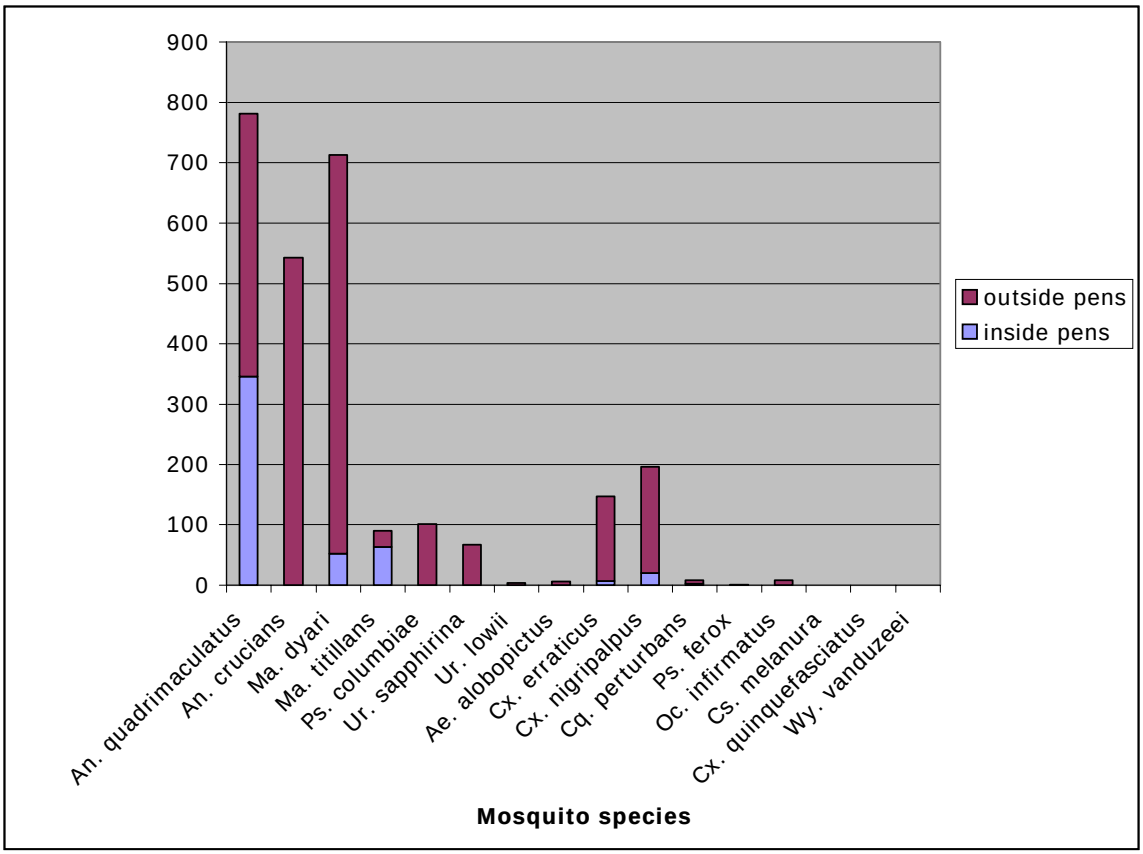


Figure 3-2. Portions of each mosquito species captured in CDC light traps set outside of alligator pens versus inside of pens (for collecting trips 1,2, and 4).

**Trip one.** A total of 2665 mosquitoes was collected from CDC traps (Table 3-1) during trip one, and this represented more than half of the total collected during the four trips.

**Trip two**. A total of 150 mosquitoes was collected during this trip (Table 3-2). Of the four CDC light traps inside of alligator pens, one was damaged by the alligators. The collection bag was removed from the trap and pushed into the water of the alligator pen, thus no mosquitoes were obtained from that CDC trap.

**Trip three.** A total of 628 mosquitoes was collected (Table 3-3). The pen or position were samples were collected can not be stated with certainty because the records of trap placement were lost while in transit between Farm A and Gainesville.

Table 3-1. Mosquitoes captured from CDC light traps during Trip one at Farm A. Numbers in [ ] indicate the number blood-fed mosquitoes.

| | CDC inside pen | | CDC outside pen | | TOTAL |
|---|---|---|---|---|---|
| | trap 1 | trap 2 | trap 1 | trap2 | |
| *An. quadrimaculatus* | 343 | 3 | 184 | 251 | 781 |
| *An. crucians* | 0 | 0 | 179 | 364 | 543 |
| *Ma. dyari* | 46[1] | 6[1] | 323[3] | 338 | 338 |
| *Ma. titillans* | 63[2] | 0 | 4 | 23 | 27 |
| *Ps. columbiae* | 0 | 0 | 39 | 62 | 101 |
| *Ur. sapphirina* | 0 | 0 | 28 | 39 | 67 |
| *Ur. lowii* | 0 | 0 | 3 | 1 | 4 |
| *Ae. alobopictus* | 0 | 0 | 0 | 6 | 6 |
| *Cx. erraticus* | 7 | 0 | 56 | 84 | 147 |
| *Cx. nigripalpus* | 19 | [1] | 54[1] | 122[1] | 19 |
| *Cq. perturbans* | 2 | 0 | 5 | 1 | 8 |
| *Ps. ferox* | 0 | 0 | 0 | 1 | 1 |
| *Oc. infirmatus* | 0 | 0 | 0 | 8 | 8 |
| *Cs. melanura* | 0 | 0 | 0 | 0 | 0 |
| *Cx. quinquefasciatus* | 0 | 0 | 0 | 0 | 0 |
| *Wy. vanduzeei* | 0 | 0 | 0 | 0 | 0 |
| TOTAL | 480 | 10 | 875 | 1300 | 2665 |

Table 3-2. Mosquitoes collected in resting boxes and CDC light traps during the second collecting trip to Farm A. Numbers in [ ] indicate blood-fed mosquitoes.

| | CDC inside pen | | | | |
|---|---|---|---|---|---|
| | trap 1 | trap 2 | trap 3 | Resting box | TOTAL |
| *An. quadrimaculatus* | 0 | 9 | 25 | 3 | 37 |
| *An. crucians* | 0 | 0 | 8 | 3[1] | 8 |
| *Ma. dyari* | 2 | 7 | 22 | 0 | 31 |
| *Ma. titillans* | 4 | [2] | 40 | 0 | 44 |
| *Ps. columbiae* | 0 | 0 | 0 | 0 | 0 |
| *Ur. sapphirina* | 0 | 0 | 0 | 0 | 0 |
| *Ur. lowii* | 0 | 0 | 0 | 0 | 0 |
| *Ae. alobopictus* | 0 | 0 | 0 | 0 | 0 |
| *Cx. erraticus* | 4 | [1] | 0 | [2] | 4 |
| *Cx. nigripalpus* | 12 | 3 | 3 | 0 | 18 |
| *Cq. perturbans* | 0 | 0 | 0 | 0 | 0 |
| *Ps. ferox* | 0 | 0 | 0 | 0 | 0 |
| *Oc. infirmatus* | 0 | 0 | 0 | 0 | 0 |
| *Cs. melanura* | 0 | 0 | 0 | 0 | 0 |
| *Cx. quinquefasciatus* | 0 | 0 | 0 | 0 | 0 |
| *Wy. vanduzeei* | 0 | 0 | 0 | 0 | 0 |
| TOTAL | 22 | 22 | 98 | 8 | 150 |

Table 3-3. Mosquitoes collected from CDC light traps and resting boxes during the third collecting trip to Farm A. Numbers in [ ] indicate blood fed individuals.

| | CDC trap | | | | | | Resting | TOTAL |
|---|---|---|---|---|---|---|---|---|
| | trap 1 | trap 2 | trap 3 | trap 4 | trap 5 | trap 6 | box | |
| *An. quadrimaculatus* | 7 | 9 | 36 | 6 | 7 | 109 | [9] | 183 |
| *An. crucians* | 2 | 9 | 30 | 0 | 0 | 0 | 0 | 41 |
| *Ma. dyari* | 4 | 0 | 58 | 3 | 4 | 39[3] | 0 | 108 |
| *Ma. titillans* | 0 | 5 | 3 | 2 | 12 | 46 | 0 | 68 |
| *Ps. columbiae* | 0 | 2 | 3 | 0 | 0 | 0 | 0 | 5 |
| *Ur. sapphirina* | 5 | 9 | 35 | 0 | 0 | 0 | 0 | 49 |
| *Ur. lowii* | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 2 |
| *Ae. alobopictus* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 1 |
| *Cx. erraticus* | 9 | 10 | 50 | 0 | 0 | 1 | 0 | 70 |
| *Cx. nigripalpus* | 20 | 19 | 40 | 0 | 0 | 5 | 8 | 92 |
| *Cq. perturbans* | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 2 |
| *Ps. ferox* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Oc. infirmatus* | 0 | 0 | 7 | 0 | 0 | 0 | 0 | 7 |
| *Cs. melanura* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Cx. quinquefasciatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Wy. vanduzeei* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| TOTAL | 48 | 63 | 265 | 11 | 23 | 201 | 17 | 628 |

**Trip four.** On the last collecting trip 1041 mosquitoes were collected (Table 3-4). The battery failed on one of the CDC traps although some mosquitoes were still collected.

Numbers and proportions of different mosquito species varied from one collecting trip to the next, however, statistical analysis of these differences was not done as the sampling size and system did not allow it.

## Blood Meal Identification

The Crocodilian-specific primers produced a PCR product band of the correct size for six of the 37 blood-fed mosquito samples (two *Cx. erraticus* and four *Ma. dyari*). Of

these six positives, one was a mosquito from a resting box and the others were from CDC traps. There were also DNA bands of the wrong size (about 180 bp) for two

Table 3-4. Mosquitoes captured in CDC light traps and resting boxes on the fourth collecting trip to Farm A. Numbers in [ ] indicate blood-fed individuals.

| | CDC inside pen | | | CDC outside pen | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | trap 1 | trap 2 | trap 3 | trap 1 | trap 2 | trap 3 | trap 4 | Resting box | TOTAL |
| *An. quadrimaculatus* | 3 | 18 | 0 | 20 | 51 | 51 | 3 | 25[6] | 171 |
| *An. crucians* | 1 | 0 | 0 | 28 | 54 | 41 | 15 | 3 | 142 |
| *Ma. dyari* | 0 | 2 | 2 | 152 | 102 | 30 | 30 | 0 | 318 |
| *Ma. titillans* | 22 | 10 | 2 | 12 | 1 | 12 | 9 | 0 | 68 |
| *Ps. columbiae* | 0 | 0 | 0 | 1 | 1 | 6 | 1 | 0 | 9 |
| *Ur. sapphirina* | 0 | 0 | 0 | 3 | 16 | 3 | 4 | 0 | 26 |
| *Ur. lowii* | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 2 |
| *Ae. alobopictus* | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 |
| *Cx. erraticus* | 1 | 0 | 0 | 9 | 29 | 6 | 2 | 2 | 49 |
| *Cx. nigripalpus* | 0 | 0 | 0 | 43 | 148[1] | 27 | 10 | 1 | 229 |
| *Cq. perturbans* | 1 | 0 | 0 | 7 | 2 | 0 | 0 | 0 | 10 |
| *Ps. ferox* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Oc. infirmatus* | 0 | 0 | 0 | 6 | 0 | 0 | 2 | 0 | 8 |
| *Cs. melanura* | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 0 | 3 |
| *Cx. quinquefasciatus* | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 2 |
| *Wy. vanduzeei* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| TOTAL | 28 | 30 | 4 | 284 | 405 | 179 | 79 | 32 | 1041 |

*Cx. nigripalpus* and one *Ma. titillans* (the *Ma. titillans* had a second, very faint band of approximately the correct size). Sequences from the correct-sized bands matched that of the positive control (193 out of 200, or 96.5% homology). The other bands produced sequences that matched neither the positive control nor any entry on the GenBank database.

Of the 37 mosquitoes that had apparent blood meals, 14 reacted with one of the mitochondrial primer sets (mammal, alligator, bird) giving an identification rate of 37.8%. Seven individuals (representing three species) were positive for alligator DNA (Fig. 3-3), six (three species) were positive for mammalian DNA, and one individual was

positive for avian DNA (Fig. 3-4). For the alligator primer set, the seven positives included the six samples that were positive for the Crocodilian primer set and the seventh was the *Ma. titillans* for which the Crocodilian primers had produced two bands, the fainter of which was the appropriate size for a positive.

Sequencing confirmed that all seven of the alligator-positive PCR bands were from *Alligator mississippiensis*. The mosquitoes feeding on alligators were *Cx. erraticus* (two individuals), *Ma. dyari* (four individuals), and *Ma. titillans* (one individual). All of these individuals except for one *Cx. erraticus* were obtained from CDC traps that were inside of alligator pens. The exception was from a resting box (Table 3-5).

Sequencing allowed species identification of the mammalian and avian blood meals. The single avian positive was from a *Cx. nigripalpus* that had fed on a turkey, (*Meleagris gallopavo*). For the mammalian positives, one *Cx. nigripalpus* fed on a raccoon (*Procyon lotor*), two *An. quadrimaculatus* fed on pigs (*Sus scrofa*), one *Ma. dyari* fed on a pig, and another *Ma. dyari* fed on a horse (*Equus callabus*). The blood meal of one *An. quadrimaculatus* could not be identified further (Table 3-5). The GenBank E value for all of the matches was 0.0 except for the match with the raccoon, where the E value was $5e^{-175}$ (indicating that there is zero or almost zero probability that these matches were due to chance). Sequences are in Appendix E.

The mammalian-specific primers consistently amplified mosquito DNA in the negative control (an un-engorged mosquito). Repetition of the DNA extraction from a new un-engorged mosquito reduced the possibility of contamination of the negative control with mammal DNA. The sequence of the brightest DNA PCR fragment did not match closely with any of the GenBank entries, however one 64 bp portion of the region

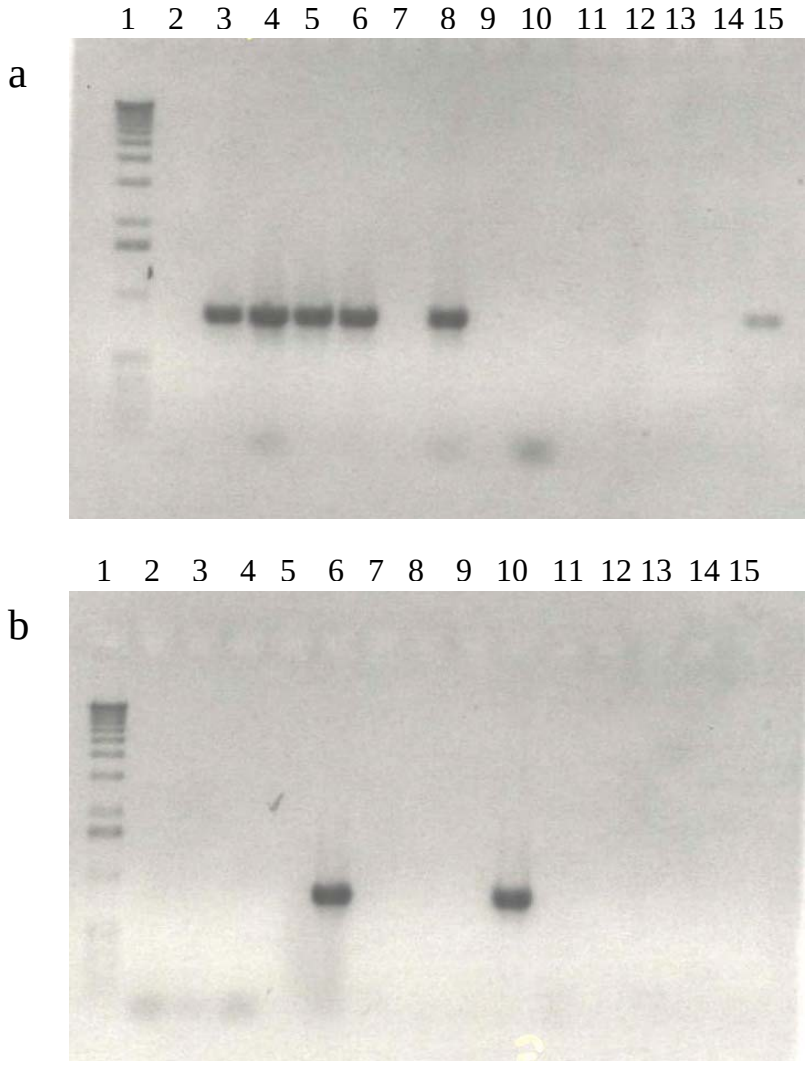


Figure 3-3. Products from PCR amplification of mosquito samples with alligator-specific primers. Gel a: lane 1 contained a 1 kb ladder, lanes 3,4, and 8 contained *Ma. dyari*, lanes 5 and 6 contained *Cx. erraticus*, and lane 15 contained the alligator positive control. Gel b: lane 1 contained a 1 kb ladder, lane 6 contained *Ma. dyari*, and lane 10 contained *Ma. titillans*.

(out of the 422 bp sequence) did match closely with chromosomal DNA (from partial mRNA) from *An. gambiae*. Within this 64 bp portion there were 57 bases shared with the database's *An. gambiae* sequence and the E-value assigned for this match was $3e^{-10}$.

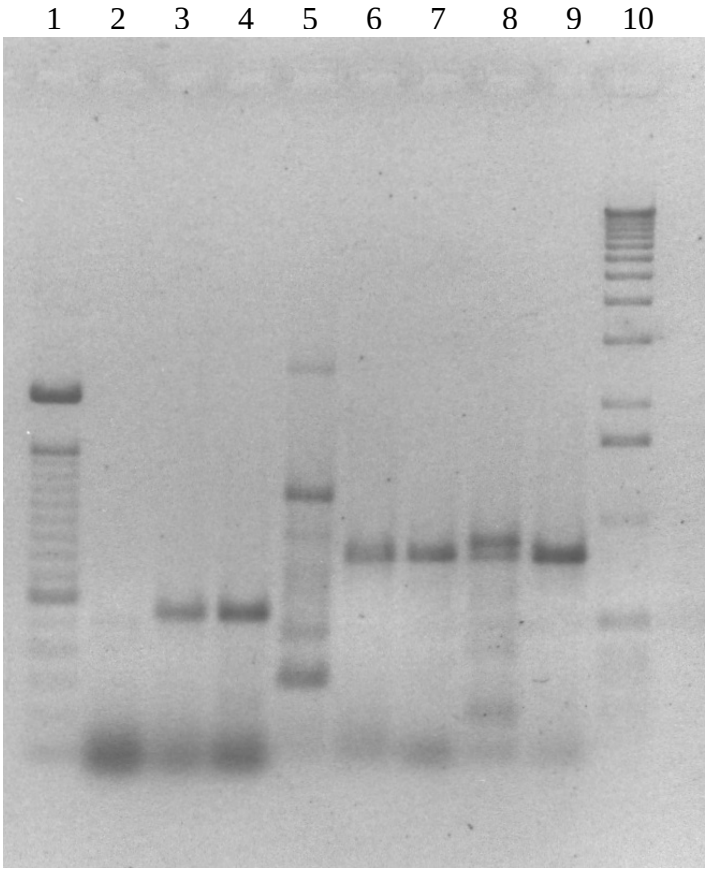


Figure 3-4. Products from PCR amplifications with a mammalian-specific primer set (lanes 2-5) and an avian-specific primer set (lanes 6-8).

Sequencing results from the correct-sized bands often had multiple overlapping peaks, suggesting that the mammal primers may sometimes bind and amplify a portion of the chromosomal DNA of mosquitoes. When one of these bands was cloned into bacteria, only some of the clones were of fragments whose sequences matched with vertebrates. The other clones yielded sequences for which there were no close matches on the database.

Table 3-5. Identities of vertebrate hosts as determined by sequencing the PCR product, and information about collection date and location on farm of the mosquito sample.

| Primer set | Mosquito | | Vertebrate host | Collection date | Trap location |
|---|---|---|---|---|---|
| | Species | ID number | | | |
| Mammal | | | | | |
| | ***Ma. dyari*** | 1MBF4 | *Equus callabus* (horse)[a] | Sept. 12 | Inside pen |
| | | 1MBF1(2) | *Sus scrofa* (wild boar)[b] | Sept. 12 | In trees on West margin |
| | *An. quadrimaculatus* | 3AnBF | *Sus scrofa* | Oct. 17 | Resting box |
| | | 3An3BF | *Sus scrofa* | Oct. 17 | Resting box |
| | | 3An8BF | *** | Oct. 17 | Resting box |
| | *Cx. nigripalpus* | 4CuNBF | *Procyon lotor* (raccoon)[c] | Oct. 24 | Eastern margin on tree |
| Bird | | | | | |
| | *Cx. nigripalpus* | 1CuNBF2 | *Meleagris gallopavo* (turkey)[d] | Sept. 12 | In trees on West margin |
| Alligator | | | | | |
| | *Cx. erraticus* | | *Alligator mississippiensis*[e] | | Resting |
| | | 2ABF | | Sept. 26 | box |
| | | 2CBF | *Alligator mississippiensis* | Sept. 26 | Inside pen |
| | *Ma. dyari* | 2DBF | *Alligator mississippiensis* | Sept. 26 | Inside pen |
| | | 1MBF3 | *Alligator mississippiensis* | Sept. 12 | Inside pen |
| | | 3AABF | *Alligator mississippiensis* | Oct. 17 | CDC 8 |
| | | 3AAABF | *Alligator mississippiensis* | Oct. 17 | CDC 8 |
| | *Ma. titillans* | 2MaTBF2 | *Alligator mississippiensis* | Sept. 26 | Inside pen |

References for identification of sequences: [a] = GenBank accession # D32190 (Chikuni, 1994); [b] = AY237534 (Alves et al., 2003); [c] = U12853 (Lento et al., 1995); [d] = L08381 (Kornegay et al., 1993); [e] = AF318572 (Glen et al., 2002), *** = specific host not identified.

## Virus Detection

**Isolation of virus in cell cultures.** In cell culture two of the three WNV positive control wells showed obvious CPE before day seven. For the direct inoculation (MOI = 32), strong CPE was apparent on day two. For the positive control with MOI = 0.63, small foci of infection were observed on day three and by day five infected cells were apparent through out the well. In these two positive controls, all cells appeared infected by day seven and many had detached from the substrate and were floating in the media. No CPE was apparent in the well that received the inoculum with MOI = 0.0063.

No apparent viral CPE was observed in any of the wells containing homogenate from field-collected mosquitoes. This and the absence of positive bands from RT-PCR indicated that there were no WNV positive field-collected mosquito pools, giving an MIR of 0/4447.

Two of the wells had bacterial contamination that became apparent after two days. When these samples were repeated (as described above) one had bacterial contamination again, and the other did not.

The cells in many of the wells that had been inoculated with homogenate from a pool of 50 *Ma. dyari* showed some effects that were believed to be due to non-viral components of the mosquitoes. The cells did not grow as well in the center of the well, and many had a “lacey” appearance, i.e. the cells appeared to have more vacuoles and margins of the cells became less smooth. However this condition was not progressive, and there were still many healthy cells in the well at day seven. The addition of 100 µl of cysteine to the diluent prior to homogenization appeared to prevent this effect in subsequent pools of 50 *Ma. dyari*.

**Detection of viral RNA.** Both of the positive controls that were tested for WNV RNA showed clear, bright positive bands with both the screening primers (about 300 bp) and the confirmation primers (about 1000 bp) (Fig 3-5). The results from the positive

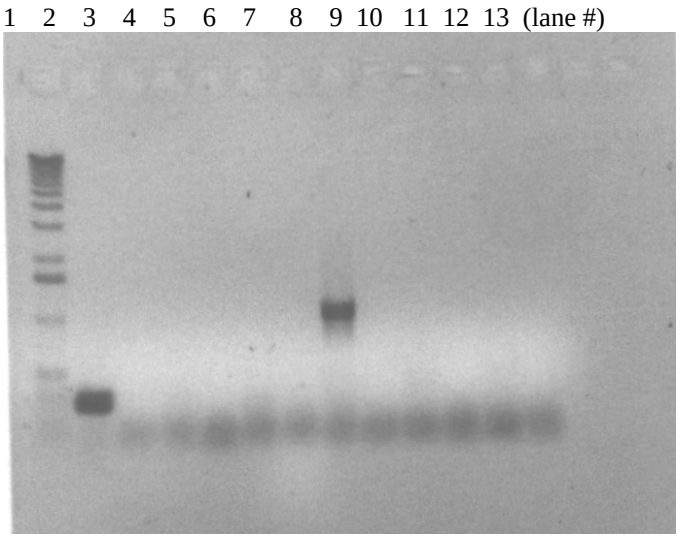


Figure 3-5. Products from RT-PCR with WNV screening primer set (lanes 2-7) and WNV confirmation set (lanes 8-13). WNV positive controls are in lanes 2 and 8. Lane 1 contains a 1 kb molecular weight standard ladder. Lanes 3 and 10 were no-template negative controls. All other lanes contain samples (4C7, 4G7, 4A3, and 4L1) for which there was some amplification in the original screening, but not in any subsequent RT-PCR reactions.

controls demonstrated that the cell culture screening was sensitive enough to detect mosquitoes containing a "normal" titer of WNV (Johnson et al., 2003), but may have missed mosquitoes with the minimum titer of $10^5$. However RT-PCR would have detected infected mosquitoes with more sensitivity, including those with titers well below $10^5$. No field-collected mosquito pools were positive for WNV RNA.

# CHAPTER 4
# DISCUSSION

## Blood Meal Identification

While encouraging, the results from the Crocodilian-specific primers (Yau et al. 2002), did not seem conclusive, because the primers were not specific. Because there are mitochondrial sequences for many more different organisms published and available on the GenBank database, it is easier to identify an unknown mitochondrial sequence than an unknown chromosomal sequence. The database contains chromosomal DNA entries for fewer reptiles and nothing was known about the region of DNA that the Crocodilian-specific primers amplified, therefore it was difficult to determine whether or not the homology found between the mosquito samples and the alligator positive control constituted a real match. It was these unknowns that led to the selection of the second primer set, one designed to amplify alligator mitochrondrial DNA from a well-studied region, the cytochrome b gene.

It appeared that the mammalian-specific primers sometimes amplified chromosomal DNA from the mosquito. The bands in the negative control (where only mosquito DNA was present) shared some homology with a mosquito and even where there was vertebrate host DNA present, sequencing results (overlapping peaks and cloned fragments whose sequences were not from a known region of vertebrate DNA) showed that the primers annealed in multiple places apparently on both host and mosquito DNA.

Of the three mosquito species found to feed on alligators, only one, *Cx. erraticus*, has been reported to feed on reptiles and in general has been identified as an

opportunistic feeder taking meals from mammals, birds, and reptiles or amphibians (Robertson et al., 1993; Irby and Apperson, 1988). Species in the genus *Mansonia* are in general considered mammal and some times bird feeders (Edman, 1971). Studies of this genus in other parts of the world have found them attracted to or feeding on cow and human "baits" (Tuno et al., 2003; Khan et al., 1997). The normal feeding habits of *Ma. titillans* and *Ma. dyari* (both of the neotropical subgenus *Mansonia*) have not received much attention probably because they do not seem to be implicated in transmission of pathogens in the United States. While *Mansonia* do not appear to be reptile feeders, this would not be the first instance of feeding "patterns" being strongly influenced by availability of hosts. Edman (1971) found that the number of mosquitoes with squirrel blood meals increased dramatically on a night when caged squirrels happened to be placed near the collection site. On Farm A, thousands of alligators are captive in pens with water depths insufficient to allow the alligators to submerge. They may present a blood source so readily available that a range of species takes advantage.

The mitochondrial PCR product from the alligator positive control was distinctly fainter than the bands from the field collected mosquitoes. This may have been due to the presence of heparin in the positive control alligator blood. Yokota et al. (1999) found that heparin interfered with PCR when template DNA was from heparinized blood, and the degree of interference was related to the concentration of heparin and the type of polymerase enzyme used.

The total blood meal host identification percentage (38%) was lower than that of other studies (65%) where host determination was done using DNA probes and a PCR reaction (Ngo and Kramer, 2003; Leslie Rios, personal communication). This may be

due to components within the mosquito or processes during digestion that interfere with the PCR or rapidly degrade the DNA. Cupp et al. (2004) found that their overall identification percentage (for two *Culex* species) was 65%, but was much lower for *Ur. sapphirina* with only two individuals out of the 35 (about 6%) blood fed collected yielding a result. While *Uranotaenia* are quite small mosquitoes, it seems unlikely that the size of the blood meal alone would be responsible for the dramatically smaller identification rate, especially considering that smaller blood meals (incomplete engorgements in "normal" sized mosquito species) were successfully amplified in this study and that in other studies there was no negative correlation between blood meal size and success of amplification (Mukabana et al., 2002). In a study working only with *An. gambiae*, Gokool et al. (1993) had a 31% positive identification rate. For this study, when Anopheline and Culicine mosquitoes are considered separately, the identification rates are 18.8% and 52% respectively. The idea that differences in mosquito digestive physiology might influence the success rate of PCR-based host identification studies warrants further study. After a blood meal is ingested it clumps inside the mosquito midgut yielding separated serum and a clot containing the erythrocytes. After that (and for the next several hours) enzymes are secreted which begin to digest the surface of the clot. Components of the separate serum are absorbed and used for nutrition or egg development (Nayar and Sauerman, 1977). Nayar and Sauerman (1977) showed that in *An. quadrimaculatus*, the mean clotting time was significantly greater than that of five Culicine mosquitoes. The average clotting time (based on results from five different blood hosts) was 203 minutes for *An. quadrimaculatus*, compared with 45, 40, 31, 21, and about 8 minutes for *Ae. taeniorhynchus*, *Oc.. sollicitans*, *Ae. aegypti*, *Ps. columbiae*,

and *Cx. nigripalpus*, respectively. The delay in clotting (likely due to differences in salivary anticoagulants) may allow enzymes to more readily reach and digest the erythrocytes in an *Anopheles* blood meal. In addition, *An. quadrimaculatus* blood is sometimes excreted within a few hours of feeding (Nayar and Sauerman, 1977). The net effect of these differences may result in host DNA being degraded more quickly in *Anopheles* than in some other mosquito genera.

In this study mosquito collections were gathered and placed on ice in the early afternoon. Assuming mosquitoes were active and thus captured at dusk (Bidlingmayer, 1967), then many of the blood fed individuals would have been placed on ice about 18 h after they had taken a blood meal (estimated dusk at 9:00 PM). In other studies (Cupp et al., 2004; Ngo and Kramer, 2003) mosquitoes were collected at dawn, or about 10 h after taking a dusk blood meal. The additional eight h of time may have allowed greater breakdown of DNA, thus making the positive identification percentage lower in this study.

**Virus Detection**

Since there were no virus isolations and no WNV RNA detected in mosquito pools, the MIR was 0 in 4447 or 0 in 270, 268, and 1161 for *Cx. erraticus*, *Ma. titillans*, and *Ma. dyari* (the three species that fed on alligators). The virus isolation results neither support nor diminish the possibility of mosquito-transmitted WNV on the alligator farm, nor can they help in incriminating any one of the three alligator-feeding species found. In a study done in New York, WNV isolations tended to occur in the vicinity of greater transmission such that the authors made the following generalization: the greatest number of human cases and dead crows corresponded to a mosquito MIR of 5.27/1000, a few human cases and moderate number dead crows corresponded to an MIR of 0.18 to

2.36/1000, and no human cases and few dead crows corresponded to an MIR of 0 to 0.86/1000 (White et al., 2001). Some studies have had similar MIR's (Rutledge et al., 2003; Reisen et al., 2004), and others have had much lower MIR's, even when there has been evidence of WNV transmission, such as dead birds (Meece et al., 2003; Andreadis et al., 2001). The MIR for collections made in Ohio by Mans et al. (2004) was higher (8 out of 1000), but they tested only those mosquitoes collected from gravid traps, thus biasing the results towards a higher MIR by testing only older females. In a Florida study, an MIR of 1.2 in 1000 was found, and results also indicated that viral activity was very focal (Rutledge et al., 2003). In this study, almost 12,000 mosquitoes were collected and two species, *Cx. nigripalpus* and *Cx. quinquefasciatus* made up about 78% of the collection. Fourteen pools from these two species were positive for WNV, and a single *Cx. nigripalpus* was responsible for infecting a sentinel chicken. This species was present around and feeding on the host (chicken) and was most frequently infected with WNV, showing that these criteria can be helpful in identifying possible vectors. This study also found that the number of WNV positive mosquitoes around the chickens was greater than the number of transmissions to chickens. Thus an infected mosquito pool is not a sure way to identify the species responsible for transmission. Alternatively, the study found that there were no infected mosquitoes at a site where a horse had become infected a month prior to collecting. In this case, the mosquito infection rates underestimated transmission rates, probably because collecting was started after the transmission had taken place.

Many studies also found *Culex* mosquitoes to be the most frequently infected with WNV, so the MIR of these species may be more meaningful for comparisons to the

situation at the alligator farm. On the alligator farm the mosquito collections were only about 18% *Culex* (808 out of 4484). The collection was predominately (75%) *Anopheles* and *Mansonia*, genera from which WNV is much more rarely isolated. So although there were almost 4500 mosquitoes collected, an "average" MIR of 1 in 1000 could not be expected as many of the collections that this MIR is based on were dominated by *Culex* species.

Based on the above information, it appears that the current study may have "missed" any WNV positive mosquitoes on the alligator farm because: 1) the mosquito collection was not large enough, 2) the mosquito collections were predominately non-*Culex* mosquitoes which are less likely to vector and be infected with WNV or 3) the collecting began during the epidemic of disease and after transmission had occurred. With the last explanation, it is possible that the transmission of WNV had taken place about two weeks before the majority of the alligators began to die. Work by Klenk et al. (2004) showed that alligators developed viremia about 5 days after infection and that they in turn infected their tank mates about a week after that viremia developed. In one possible scenario, mosquitoes infected several alligators during a brief period of intense WNV activity. These alligators then developed viremia and infected their tank mates, and a week later the situation progressed to what was observed during the first collecting trip: multiple alligators sick and dying from WNV-like disease. In this scenario the WNV transmission was very focal (both temporally and spatially) and had subsided by the time mosquito collecting had begun. If this were the case, the practice of pre-epizootic surveillance would not only help predict when an epizootic might start, but would also be important for identifying the mosquito species involved.

Surveillance reports were used to fill in information about the state of transmission in the area around the time of the outbreaks. If the alligator epidemics were isolated, it may suggest a cause separate from the surrounding virus activity, (i.e. infection due to WNV contaminated meat). However, if there was transmission, as demonstrated by infected horses, humans, birds, or sentinel chickens, this supports the idea that the outbreaks on the alligator farms were related to the virus activity occurring in the area. The reports posted by the Florida Mosquito Control Association and the United States Geological Service's maps (http://westnilemaps.usgs.gov/index.html, created with information from CDC) give information about the level of WNV activity in the vicinity of the alligator farm during the fall of 2003. During that year, the county had 64 conversions in sentinel chicken flocks, one osprey (*Pandion haliaetus* Linnaeus) positive for antibodies to WNV, and three cases of WNV reported by veterinarians. However, there were no isolations of virus from mosquito pools in the county that year. This surveillance information indicates that there was mosquito transmission of WNV in the county during the time of the epizootic on the alligator farm, even though no isolations were made from mosquitoes. The possibility remains that the WNV on Farm A was mosquito transmitted even though no positive mosquitoes were detected.

**Vector Incrimination**

The information gained in this study can be considered in the context of the criteria established by Reeves (1954) and expanded on by Kilpatrick et al. (2005) and used to identify potential mosquito vectors. Because this study did not identify WNV in any mosquito pool or identify any competent vectors for WNV, information from other studies can be incorporated to identify potential vectors. In this study it was established that *Cx. erraticus, Ma. titillans*, and *Ma. dyari* feed on alligators at the farm and that

these species are relatively numerous around the farm (6%, 6%, and 26% of the total catch, respectively). Other studies have shown that they are in greatest abundance during the season when the alligator epidemics occur (Bidlingmayer, 1968; Zhong et al., 2003). Information from other studies and surveillance reports will be necessary to answer the remaining questions about these potential vectors: are they competent vectors for WNV, and are they repeatedly found infected with the virus?

*Culex erraticus* is a member of the neotropical subgenus Melanoconion, and is found all over the eastern United States, as far north as Connecticut and New York (Andreadis, 2003; Kulasekera et al., 2001), south of the great lakes, through out the southeast (Darsie and Ward, 1981), and has been found in California (Lorthrop et al., 1995). *Culex erraticus* specimens have been found positive for West Nile virus each year from 2002 to 2004 (CDC, http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm). They have also been found infected with other arboviruses in the United States such as Eastern Equine Encephalomyelitis virus (EEE, Togaviridae: Alphavirus) (Wozniak et al., 2001; Cupp et al., 2003) and St. Louis Encelphalitis virus (Cupp et al., 2004a). St. Louis Encephalitis (SLE) is also a flavivirus of the Japanese encephalitis (JE) serogroup (Poidinger et al. 1996). *Culex erraticus* are considered competent vectors of EEE (Cupp et al., 2004b). However, competence for one type of arbovirus, often does not correlate with competence for another (Hardy et al., 1983). Some reports have given a WNV minimum infection rate for this species (Gaines, Virginia Department of Health), although in many cases this species was pooled and/or reported together with other *Culex* species under the general heading "*Culex* sp.". This makes it difficult to know the minimum infection rate

although it is possible to say that WNV has been repeatedly isolated from these mosquitoes and that other members of the genus are the most commonly found WNV-positive mosquitoes. As of July 2005, no WNV vector competence studies have been published for *Cx. erraticus* or for any other North American Melanoconion. The studies that have been done indicate that all of the *Culex* species tested have moderate to excellent vector competence and moderate to excellent potential to vector WNV (Turell et al., 2005). Based on laboratory experience, *Cx. erraticus* appears to be a long-lived species (Klein et al., 1987), and this could contribute to its potential vector competence.

In the United States, *Ma. dyari* is found in Florida and parts of Georgia and South Carolina (Darsie and Ward, 1981; Darsie and Hager, 1993). *Mansonia titillans* has been found in central and south Florida, in southern Texas, and in Mississippi (Darsie and Ward, 1981; Goddard and Harrison, 2005). These species are also found in south and Central America, where *Ma. titillans* is likely involved in the transmission of Venezuelan Equine Encephalomyelitis virus, an alphavirus (Mendez et al., 2001; Turell et al., 2000) and *Ma. dyari is* a maintenance vector of SLE (Gorgas Memorial Laboratory 1979, as cited in Lounibos et al., 1990). As with *Cx. erraticus*, no vector competence studies with WNV have been done for *Mansonia* species. WNV has been detected in pools of several species of *Mansonia* in Africa (Traoré-Lamizana et al., 2001), and other members of the genus appear to be involved in transmission of JE in Asia (Arunachalam et al., 2002; Arunachalam et al., 2004). Regardless of their presumed vector competence based on these other diseases, *Ma. dyari* has never been positive for WNV in the United States and WNV has been detected in *Ma. titillans* only in 2004 (http://www.cdc.gov/ncidod/dvbid/westnile/mosquitoSpecies.htm). Based on this input,

*Cx. erraticus* seems the most likely potential vector of WNV on Farm A, although *Mansonia* are at least nuisance species.

**Mosquito Control**

*Culex erraticus*, *Ma. dyari*, and *Ma. titillans* are all associated with vegetated aquatic habitats (Alfonzo et al., 2005; Lounibos et al., 1990). *Culex erraticus* females prefer to oviposit where there are aquatic plants (Klein et al., 1987) and all *Mansonia* larvae are associated with aquatic plants from which they derive oxygen and possibly cover from predation (Lounibos et al., 1990). Conceivably the numbers of these mosquitoes could be controlled on Farm A by reducing the amount of aquatic vegetation present in the bodies of water. Especially with the *Mansonia*, mosquito populations are closely related to the availability of the preferred larval host plants (Lounibos and Esher, 1985), which are water lettuce, *Pistia* sp. for *Ma. dyari*, and common water hyacinth, *Eichhornia crassipes* (Mart.), for *Ma. titillans* (Slaff and Haefner, 1985). These plants can be controlled with herbicides (Slaff and Haefner, 1985). The practicality of serious water plant control in this case would need to be investigated. First, both *Ma. titillans* and *Cx. erraticus* have been reported as traveling greater than 2 km in mark and recapture studies (Morris et al., 1991), so control may have to include all vegetated water bodies within a 1 to 2 km radius. Second, farmers would need to consider the risks associated with managing vegetation in water bodies that are occupied by a number of large alligators, especially because these alligators are accustomed to receiving food from humans. Alternatively, control could be aimed at adult mosquitoes. For all three species, adulticides could be applied for several months in the late summer and early fall when populations are at their peak (Slaff and Haefner, 1985; Bidlingmayer, 1968; Roberson et al., 1993; Zhong et al., 2003).

**Alternative Vertebrate Reservoirs**

The other blood meal hosts (pigs, a horse, a turkey, and a raccoon) identified on the farm were probably not involved in maintenance or amplification of WNV. In a study where three-week-old turkeys were inoculated subcutaneously with NY99 WNV, none displayed illness, and viremia, while detectable, was very low. From the results researchers concluded that turkeys would not be severely effected by WNV nor would be they important amplification hosts (Swayne et al., 2000). In another study, pigs were subjected to mosquitoes infected with New York 99 strain of West Nile virus, and while adult pigs seroconverted, most of the animals did not have sufficient viremia to allow reisolation of the virus from serum. Weanling pigs developed viremia less than or equal to $10^{3.1}$ PFU/mL. No signs of clinical disease were observed (Teehee et al., 2005). The low viremia found in horses and their failure to infect mosquitoes in experiments also makes them unlikely amplification hosts (Bunning et al., 2002).

# CHAPTER 5
# CONCLUSIONS AND AREAS FOR FURTHER STUDY

In conclusion, the study found that of the 16 species collected in CDC light traps and resting boxes on Farm A, three species contained blood from alligators: *Cx. erraticus*, *Ma. dyari*, and *Ma. titillans.* Based on its known feeding habits *Cx. erraticus* would also feed on birds near the farm (Roberson et al., 1993). If *Cx. erraticus* has vector competence similar to what has been found for many of the other members of its genus (Turrel et al., 2005), then it could serve as a vector of WNV to alligators on Farm A. Additional laboratory and fieldwork, such as vector competence studies and efforts to screen for WNV, can further clarify the potential role of this species in WNV transmission on alligator farms. In addition, this study found *Mansonia* mosquitoes feeding on alligators, and this appears to be the first report of these two species of mosquito feeding on reptiles.

It may also be interesting and informative to study mosquitoes' responses to potentially attractive or repellent compounds associated with the alligators. In one trap placed inside of an alligator pen there were over 400 mosquitoes collected in one 24 h period, suggesting that the mosquitoes were attracted to compounds coming from the pen. A 1 kg alligator at rest should excrete about 7 mL of $CO_2$ per min (Farmer and Carrier, 2000). An alligator pen containing 200 such individuals could be putting out $CO_2$ at a rate of about 1400 mL/min, thus representing a very strong attractant for many mosquito species (Kline and Mann, 1998). However, not all of the species that were found inside of the alligator pens had blood meals from alligators. This may mean that there are

repellents or missing attractants (or other stimuli) that prevent feeding in many of the mosquitoes that were initially attracted to the alligator pens. Additional collections that are carried out more regularly and systematically may provide more information about what mosquitoes are attracted to the alligators, and whether or not these mosquitoes proceed to feed. Traps that do not have a $CO_2$ bait could be set in the alligator pens to single out species of mosquito attracted to the alligators in the absence of additional attractants. Also laboratory experiments with an olfactometer (McKenzie, 2003) could be used to determine the attractiveness of different aromatic compounds present in alligator hide to mosquitoes, thus further adding to our understanding of how mosquitoes respond to alligators as a potential blood host. The collecting results suggested that some species are more inclined to enter alligator pens than others. Investigating these differences could not only help predict vectors of WNV in alligators, but could also be useful in the continued effort to describe mosquito host seeking behavior.

# APPENDIX A
# PROTOCOL FOR QIAGEN QIAQUICK SPIN KIT, PURIFICATION OF DNA FROM AGAROSE GEL

(modified from QIAquick Spin Handbook, S. C. Garrett)

1. Add 96-100% ethanol to buffer PE before beginning.
2. Weigh the excised piece of agarose gel containing the PCR product and place in a 1.5 mL microfuge tube.
3. Add 3 μl of buffer QG for each 1 mg of gel.
4. Incubate at 50°C for 10 min (tapping tube to mix every 2 minutes) to dissolve gel.
5. Once the gel is dissolved add 1 μl of isopropanol for each 1 mg of gel and mix.
6. Place a QIAquick spin column into a 2 ml plastic collection tube.
7. Transfer the dissolved gel solution to the column and centrifuge at 13,000 rpm for one min in a microcentrifuge.
8. Remove the column, discard the flow-through from the collection tube, and place the column back into the tube.
9. Add 0.5 ml of buffer QG to the column and centrifuge for one min (13,000 rpm).
10. Discard the flow-through and return column to tube.
11. Add 0.75 ml of buffer PE and centrifuge for one min. (13,000 rpm).
12. Discard flow-through, return column to tube, and centrifuge for an additional minute at the same speed.
13. Transfer the column to a clean, labeled microcentrifuge tube.
14. Add 30 μl of buffer EB to the center of the column membrane (white material in the center of the column), allow the buffer to soak in for one min, and then centrifuge for 1 min (13,000 rpm).
15. Eluted DNA can be stored at 4°C until needed for sequencing or other purposes.

APPENDIX B

ABI PRISM™ DYE TERMINATOR CYCLE SEQUENCING KIT, PROTOCOL FOR DNA SEQUENCING

(Modified from PERKIN ELMER PROTOCOL, revised July, 2005)

NOTE: Keep all reagents on ice

1. Estimate concentration of template DNA by running 5 µl in an agarose gel and comparing intensity to known concentration of molecular weight ladder.
2. Calibrate the thermocycler.
3. Dilute templates to recommended concentration (See table below).
4. Remove Terminator Ready Reaction Mix and ICBR dNTP mix from freezer and thaw on ice.
5. For each reaction, mix the following reagents in a microfuge tube:

| **REAGENT** | **QUANTITY** |
|---|---|
| Terminator Ready Reaction Mix | 2.0 µL |
| ICBR dNTP mix | 2.0 µL |
| Template | |
| single-stranded DNA (100ng/ul) | 50-100 ng |
| double-stranded DNA (500 ng/ul) | 200-500 ng |
| PCR products (100-200 bp) | 1-3 ng |
| (200-500 bp) | 3-10 ng |
| (500-1000 bp) | 5-20 ng |
| (1000-2000 bp) | 10-40 ng |
| (> 2000 bp) | 40-100 ng |
| Primer (3.5 pmol) | ___ µL |
| Deionized Water | Bring final volume to 10.0 µL |
| Final Reaction Volume | **10.0 µL** |

6. Gently pipette to mix the reagents.
7. Place tubes in thermocycler and start thermocycle (See below).

## **Thermocycle program:**

1. Ramp to 96$^{o}$C and hold for 30 s (denaturation)
   Ramp to 50$^{o}$C and hold for 15 s (primer annealing)
   Ramp to 60$^{o}$C and hold for 4 min (product extension)
2. Repeat step 1. For 25 cycles

3. Ramp to 4$^{o}$C and hold.

8. Remove tubes from the thermocycler.
9. For each reaction, prepare a 1.5 mL microfuge tube by adding:
   1.0 µL 3M Sodium acteate, pH 4.6
   30.0 µL 95% cold ethanol
10. Transfer the sample to the prepared microfuge tube and place on ice for 10 min.
11. Centrifuge for 15 min (13,000 rpm).
12. Carefully and completely remove ethanol solution, without disturbing the pellet of DNA.
13. Rinse the pellet with 250 µL of 70% ethanol.
14. Centrifuge for 1 min to secure the pellet onto the bottom of the tube.
15. Carefully remove the 70% ethanol without disturbing the pellet of DNA.
16. Dry under vacuum.
17. Store in dark freezer until ready to read.

# APPENDIX C
# PROTOCOL FOR PGEM®-T VECTOR LIGATION KIT,

(modified from the Promega pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual, S. C. Garrett, July 2005)

1. Estimate the concentration (ng/μl) of the PCR product to be cloned by comparing the intensity of the band in a gel to the intensity of the standardized bands of the molecular weight ladders with known DNA concentrations.
2. Calculate the amount (in ng) of PCR product needed for the amount of pGEM-T vector by using the following equation:
   (ng of vector)(kb size of insert) = x ng of PCR product
3. Based on the above calculations/estimations, calculate the volume of PCR product that should be added to the vector.
4. Centrifuge the pGEM®-T vector for 4 s to concentrate contents at the bottom of the tube.
5. Vortex the 2X rapid ligation buffer before use.
6. Combine the following in a 0.5 ml microfuge tube:
   5 μl 2X rapid ligation buffer
   1 μl pGEM®-T vector
   x μl PCR product
   1 μl T4 DNA ligase
   deionized water to a final volume of 10 μl
7. Mix the reagents.
8. Incubate the mixture for 1 h at room temperature or overnight at 4°C if less than the recommended amount (see equation in step 2) of PCR product was added.

## APPENDIX D
## PROTOCOL FOR QIAPREP SPIN MINIPREP KIT, EXTRACTION OF PLASMID

(modified from QIAprep Miniprep Handbook, S. C. Garrett, July 2005)

1. Centrifuge three to five ml of bacteria from overnight bacterial culture.
2. Add RNAase A to Buffer P1.
3. Resuspend pelleted bacterial cells in 250 μl of buffer PI and transfer to a 1.5 ml plastic microcentrifuge tube.
4. Add 250 μl of buffer P2 and mix by gently inverting 4-6 times.
5. Add 350 μl of buffer N3 and mix by gently inverting 4-6 times.
6. Centrifuge the extracted DNA for 10 min at 13,000 rpm.
7. Pipette supernatant into a QIAprep column and place column into a collection tube.
8. Centrifuge for 60 s (13,000 rpm).
9. Remove column from collection tube, discard flow-through, and place column back into collection tube.
10. Add 0.75 ml of buffer PE to column.
11. Centrifuge for 60 s (13,000 rpm).
12. Discard flow-through and then centrifuge for an additional min at 13,000.
13. Transfer the column to a clean 1.5 microcentrifuge tube.
14. Add 50 μl of buffer EB to the center of the column and let the buffer soak in for one min.
15. Centrifuge for one min at 13,000 to elute DNA.

## APPENDIX E
## PROTOCOL FOR RNA EXTRACTION FROM MOSQUITO POOL USING TRIZOL LS (GIBCO)

(modified (6/7/04) and July/05 (S. C. Garrett) from Leslie Rios's protocol)

1. Homogenize mosquito pool with 1-4 beebees (copper-clad metal airgun shot) in 1ml PBS medium with 4% Fetal Bovine Serum.
2. Centrifuge at 15,000 rpm for 10 min.
3. Remove 200 μl of supernatant into a new tube and save the rest of the supernatant at -80°C.
4. To the 200 μl, add 600 μl Trizol LS, then mix and incubate at room temperature for five min.
5. Add 160 μl of chloroform, mix by inverting for about 15 seconds, and then incubate at room temperature for 15 min.
6. Centrifuge at 12,500 rpm for one min.
7. Remove the upper aqueous layer to a new tube.
8. Add isopropanol such that the isopropanol is about 0.7 times the volume of the solution then mix.
9. Centrifuge at 12,500 rpm for 15 min.
10. Pipette off the isopropanol carefully and then add 300 μl of 70% EtOH.
11. Centrifuge at 12,500 rpm for 5 min.
12. Carefully pipette EtOH and vacuum dry for 10-15 min.
13. Resuspend the dried pellet with 10 μl of RNAse-free water.

APPENDIX F
SEQUENCES OF PCR PRODUCTS USED TO IDENTIFY VERTEBRATE HOST ORIGIN OF MOSQUITO BLOOD MEALS

*Ma. dyari* (1MBF1(2)); 100% identity with *Sus scrofa* (wild boar) cyt.b gene (accession # AY237534):

ATCCGAAAATCACACCCACTAATAAAAATTATCAACAACGCATTCATTGACCTCCCAGC
CCCCTCAAACATCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCT
TGCAAATCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCT
TTCTCATCAGTTACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCT
ACATGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTC
TATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGAGTAGTCCTACTATTT
ACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTG
AGGAGCTACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCG
TAGAATGAATCTGAGGGGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCC
TTCCACTTTATCCTGCCATTCATCATTACCGCCCTCGCAGCCGTACAT

*Ma. dyari* (1MBF4); 100% identity with *Equus caballus* (horse) (accession # D32190):

TGGAATGGGATTTTGTCCATATCGGATGGGATTCCTGAGGGGTTGTTAGATCCTGTTTC
GTGAAGAAATAGTAAATGTACGACTACCAGGGCTGTGATGATGAAGGGTAGGATGAAGT
GGAAAGCAAAAAATCGGGTAAGGGTGGCTTTGTCTACTGAGAATCCACCTCAGATTCAC
TCGACGAGGGTAGTACCGATGTAGGGAATTGCTGATAGGAGGTTCGTGATGACTGTTGC
TCCTCAAAAGGATATTTGGCCTCATGGTAGGACATAGCCCATGAATGCTGTAGCTATAA
CTGTGAAAAGTAGGATGATTCCAATGTTTCATGTCTCTAGGAATGTGTAAGAGCCGTAG
TAGAGGCCGCGTCCTACGTGAATGAAGAGGCAGATAAAAAATATTGATGCTCCGTTGGC
ATGGAGGTAGCGAATAATTCATCCGTAGTTAACGTCTCGGCAGATGTGAGTGACGGATG
AGAAGGCAGTTGTCGTGTCTGATGTGTAGTGTATGGCTAGGAATAGGCC

*An. quadrimaculatus* (3AnBF), clone 2; 100% identity with *Sus scrofa* (pig) (accession # AY237534):

CCACTAATAAAAATTATCAACAACGCATTCATTGACCTCCCAGCCCCCTCAAACATCTC
ATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCTTGCAAATCCTAACAG
GCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCTTTCTCATCAGTTACA
CACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCTACATGCAAACGGAGC
ATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTCTATACTACGGATCCT
ATATATTCCTAGAAACATGAAACATTGGAGTAGTCCTACTATTTACCGTTATAGCAACA
GCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTGAGGAGCTACGGTCAT
CACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCGTAGAATGAATCTGGG

GGGGCTTTTCCGTCGACAAAGCAACCCTCACACGATTCTTCGCCTTCCACTTTATCCTG
CCATTCATCATTACCGCCCTCGCAGCCGTACATCTCCTATTCCTGCACGAAACCGGATC
C

*Cx. nigripalpus* (1CuNBF2), clone 3; 100% identity with *Meleagris gallopavo* (turkey) (accession #L08381):

CTTACTCACATTAACCCTATTCTCACCTAACCTCTTAGGAGACCCAGAAAACTTTACCC
CAGCAAATCCACTAGTAACCCCCCCACACATTAAACCAGAGTGATACTTTCTATTTGCC
TACGCAATCCTACGCTCAATCCCAAACAAACTTGGAGGTGTCCTAGCCTTAGCAGCATC
AGTACTCATTCTTCTCCTTATCCCCTTCCTTCATAAATCTAAACAACGGGCAATAACAT
TCCGGCCACTCTCACAAACCTTATTCTGACTCTTAGTAGCAAACCTCCTCATCCTAACC
TGAGTAGGAAGCCAACCAGTAGAACACCCATTCATCATCATTGGCCAAATAGCATCCCT
TTCCTACTTCACTATCTTACTAATCCTCTTCCCCTTAATCGGAGCCCTAGAAAACAAAA
TACTCAACCTCTAAGTACTCTAATAGTTTATGAAAAAC

*Cx. nigripalpus* (4CuNBF); 98.22% identity with *Procyon lotor* (raccoon) cyt.b gene (accession # U12853):

ATCCGAAAAACTCACCCATTAGCTAAAATCGTCAACAACTCATTCATTGATCTACCCAC
CCCCTCAAACATCTCAGCATGATGAAATTTCGGCTCCCTCCTCGGAATTTGTTTGCTTC
TACAGATCGCAACAGGTTTATTCTTAGCCATGCACTACACACCAGATACAGCCACAGCT
TTCTCATCAGTGACCCACATTTGCCGAGATGTAAATTATGGCTGAATTATCCGATATAT
ACACGCTAACGGAGCTTCTATATTCTTTATATGCCTATTCTTACACGTAGGACGAGGCT
TATACTATGGCTCCTATACATTCTCTGAAACATGAAATATTG

*An. quadrimaculatus* (3An3BF); 100% identity with *Sus scrofa* (accession # AY237534):

ATCCGAAAATCACACCCACTAATAAAAATTATCAACAACGCATTCATTGACCTCCCAGC
CCCCTCAAACATCTCATCATGATGAAACTTCGGTTCCCTCTTAGGCATCTGCCTAATCT
TGCAAATCCTAACAGGCCTGTTCTTAGCAATACATTACACATCAGACACAACAACAGCT
TTCTCATCAGTTACACACATTTGTCGAGACGTAAATTACGGATGAGTTATTCGCTATCT
ACATGCAAACGGAGCATCCATATTCTTTATTTGCCTATTCATCCACGTAGGCCGAGGTC
TATACTACGGATCCTATATATTCCTAGAAACATGAAACATTGGAGTAGTCCTACTATTT
ACCGTTATAGCAACAGCCTTCATAGGCTACGTCCTGCCCTGAGGACAAATATCATTCTG
AGGAGCTACGGTCATCACAAATCTACTATCAGCTATCCCTTATATCGGAACAGACCTCG
TAGAATGAATCTGAGGGGGCTTTTCCGTCGACAAAGCAACCCTCACACGA

All of the following sequences (from PCR bands of alligator mitochondrial primers) had 100% identity with *Alligator mississippiensis* sequence, accession number AF318572:

Alligator positive, bases 39-575:

ACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCT
GACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGC
TGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAA
ACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTC

CTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAAT
ATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAAC
CAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTC
TTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAAC
TGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAG
AGAACA

*Cx. erraticus* (2CBF):

ACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCT
GACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGC
TGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAA
ACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTC
CTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAAT
ATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAAC
CAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTC
TTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAAC
TGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAG
AG

*Cx. erraticus* (2ABF)

CACCCACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAA
ATGCTGACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCA
CTAGCTGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCC
TGAAAACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGT
ACTTCCTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTA
GCAATATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACA
ACAACCAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACT
TCCTCTTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGC
CAAACTGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCT
CTTAGAGAACAAAATAGTTGAACCCACCTATGTTACCCC

*Ma. dyari* (3AAABF):

CTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCTGA
CAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGCTG
CCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAAAC
TTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTCCT
ATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAATAT
TCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAACCA
ATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTCTT
ACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAACTG
CCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAGAG
AACAAAATAGTTGAACCCACCTATGTTACC

*Ma. dyari* (1MBF3):

ACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCT
GACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGC
TGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAA
ACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTC
CTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAAT
ATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAAC
CAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTC
TTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAAC
TGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAG
AGAACAAAATAGTTGAACCCACCTATGTTAC

*Ma. dyari* (3AABF):

ACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCT
GACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGC
TGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAA
ACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTC
CTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAAT
ATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAAC
CAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTC
TTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAAC
TGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAG
AGAACAAAATAGTTGAACCCACCTATGTTA

*Ma. dyari* (2DBF):

CCACCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATG
CTGACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTA
GCTGCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGA
AAACTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACT
TCCTATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCA
ATATTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACA
ACCAATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCC
TCTTACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAA
ACTGCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTT

*Ma. titillans* (2MaTBF2):

CCTGATCTTCCTCCATGAACGAGGATCATTTAACCCCCTAGGAATTAGCCCAAATGCTG
ACAAAATCCCATTCCACCCCTACTTCACCATAAAAGACGCCCTAGGAGCAGCACTAGCT
GCCTCCTCACTACTCATCTTAGCTCTCTACCTACCAGCCCTATTAGGGGACCCTGAAAA
CTTCACCCCAGCAAATTCCATAATTACCCCAACACACATCAAACCCGAATGGTACTTCC
TATTTGCTTATGCCATTCTACGATCTATTCCAAATAAGTTAGGAGGAGTACTAGCAATA
TTCTCATCCATTTTAGTCCTATTCCTAATACCCGCCCTACACACAGCAAAACAACAACC
AATATCAATACGCCCTATATCTCAGCTTCTATTTTGAGCCCTTACCCTGGACTTCCTCT
TACTCACATGAATCGGAGGCCAACCAGTAAACCCCCCATATATTTTAATTGGCCAAACT

GCCTCCCTATTCTACTTCATCATCATCCTAATCCTCATACCAATAGCAGGCCTCTTAGA
GAACAAAATAGTTGAACCCACCTATGTTAC

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# BIOGRAPHICAL SKETCH

Sandra Coral Garrett was born on the windy, cold morning of March 8, 1981 in Aiken, South Carolina, to Dr. Alfred J. Garrett and Susan Hersey Garrett. She has three siblings: an older brother Travis, a younger sister Allison, and a younger brother Benjamin. Sandra and her siblings grew up in Aiken, but also spent time on Hilton Head Island, a barrier island near the Georgia-South Carolina border. Both places presented the children with opportunity for outdoor exploration, and thus allowed Sandra to develop a strong interest in biology in addition to outdoor sports and art.

Sandra and her siblings all attended the South Carolina Governor's School for Science and Mathematics for the last two years of their high school education. This unique school and its outstanding teachers helped Sandra explore her interests in the biological sciences and prepared her for college and research pursuits. It was during a school tour of the Clemson entomology department that Sandra decided entomology might be an exciting and rewarding area of biology to study. She became interested in the University of Florida's strong entomology department and was able to attend with financial assistance from UF's National Merit Scholar program.

Sandra received a BS in entomology from UF. Experiences like her senior thesis work with Dr. Howard Frank and the Tropical Entomology field trip to Venezuela further strengthened her interest in entomology. She graduated summa cum laude from UF and decided to stay for a master's degree. She met her future husband, Dr. José Carlos V. Rodrigues, in the department and was married in May of 2005.