IDENTIFICATION OF POTENTIAL MOSQUITO VECTORS OF WEST NILE VIRUS TO HORSES IN NORTH CENTRAL FLORIDA

By

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To my husband, Salvador Rios Madrigal

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IDENTIFICATION OF POTENTIAL MOSQUITO VECTORS OF WEST NILE VIRUS TO HORSES IN NORTH CENTRAL FLORIDA

By

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West Nile virus (family *Flaviviridae,* genus *flavivirus* WNV) is of concern in the US and Florida because the virus causes disease in humans and horses. Since 1999, there have been 23,925 clinical human cases of WNV in the United States (1999-2007). Prevention and reduction of cases requires a clear understanding of the WNV transmission cycle, but much of the needed information is lacking. It is still unknown which mosquito species transmit WNV to horses. This study integrated field investigations with laboratory studies to identify possible mosquito vectors of WNV to horses in north central Florida. The primary objectives of this research were to compare the abundance and seasonality of mosquito species collected near horses, and to characterize host preference of potential vectors. An additional aim was to evaluate extrinsic risk factors of WNV to Florida horses. The extrinsic factors of interest included farm management, farm ecology, and the entomological conditions associated with each farm. A questionnaire that focused on potential risk factors was mailed to the owners of all horses tested for arbovirus from 2001 to 2003. Vaccination was the factor most strongly associated with a protective effect for WNV disease outcome in horses. The factors that were associated with an increased risk of WNV in horses were fan use in the stable, mosquito activity, and dead birds on

the property. Blood meal identification and virus screening were done in order to determine which mosquito species, if any, were involved in WNV transmission to horses. Mosquitoes were collected for a period of 26 months from a horse research area in north central Florida. DNA was extracted from the abdomen of the blood fed mosquitoes to test for the presence of avian, mammalian, and reptilian blood using PCR with different primer sets. The blood meals were confirmed with sequencing. The non-blood-fed mosquitoes were sorted into pools of up to 50 mosquitoes and screened for WNV, SLEV, and EEEV by Real-Time quantitative RT-PCR. A total of 45,851 mosquitoes (twenty three species) were collected, 252 of which had visible blood meals. Twelve mosquito species (fifty eight individuals) were positive for horse DNA. St. Louis encephalitis virus was detected in one pool of *Mansonia titillans* collected on September 26, 2006. This study was able to identify several mosquito species feeding on horses and risk factors associated with WNV disease. The vaccine can protect horses against WNV disease if administered two weeks prior to exposure and if a booster is administered yearly.

CHAPTER 1
INTRODUCTION AND REVIEW OF THE LITERATURE

Introductory Statement

In Florida, West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) continues to threaten the health of humans and domestic animals. Between 1999 and 2007, 23,925 human cases of WNV disease have occurred in the United States {2007 1905 /id}. West Nile virus is considered an emerging infectious disease (EID) in the United States (NIAID 2008). An EID is a disease that is newly recognized or a previously known pathogen that has spread in incidence or geographic range (Lederberg et al. 1992). As WNV becomes locally established, recurrent epidemics occurring seasonally in the summer through fall will likely continue to occur (Hayes et al. 2005). Florida may be especially susceptible to WNV epidemics due to the subtropical climate and the endemic status of another closely related arbovirus, St. Louis encephalitis virus (family *Flaviviridae*, genus *Flavivirus*, SLEV). Additionally, Florida plays an important role in the equine industry (FDACS 2008); many breeding horses are located within the state and are at risk of infection. The focus of this dissertation was on the ecology of WNV in Florida with respect to mosquito vectors and animal disease. Unlike other states where human infection has and now predominates, animal infection has been the most prominent feature in Florida WNV encroachment. Thus laboratory and field studies investigating the epidemiology of WNV in horses and their potential vectors in a region of high WNV activity during encroachment, north central Florida, can contribute to our overall understanding of WNV in Florida. The purpose of the following literature review is to provide a background of relevant WNV literature and present a summary of WNV surveillance and detection in Florida. The goal of this dissertation is to strengthen understanding of the WNV vector-host interactions in an area where susceptible hosts (horses) are present. The primary objectives of this research were to document the mosquito

species collected near horses, identify the seasonal distribution of these mosquito species, and characterize their host preferences. *The central testable hypothesis of this work is that microhabitats and weather conditions dictate the local mosquito species present, and some of these species may be capable of transmitting arboviruses to clinically susceptible hosts.*

Introduction

West Nile virus is an enveloped, single stranded, positive sense RNA virus in the family Flaviviridae {ICTVdB Management, 2006 19 /id}. The first isolation of WNV was from a febrile woman in the West Nile District of Uganda in 1937 (Smithburn et al. 1940). Cases of West Nile fever, (WNV infection resulting in fever, headache, and/or rash) have been regularly reported in Africa, West Asia, and the Middle East. West Nile virus was recognized as a cause of central nervous system (CNS) infections such as meningitis and encephalitis when a number of people became sick in Israel in 1951 (Work et al. 1955). In horses, summer neurological syndromes were observed since the early 1900s and equine cases caused by WNV were first identified in the early 1960s in France (Murgue et al. 2001). Since its introduction to the United States in 1999, WNV has been a growing public health concern in the western hemisphere {2008 2281 /id}. The strain of WNV introduced into New York in 1999 (NY99) was fist isolated from an American Crow (*Corvus ossifragus* Brehm). This strain was sequenced and found to have over 98% homology with a WNV strain isolated from a goose in Israel in 1998 (Brinton 2002). Since its introduction in the United States, WNV has spread throughout the US, Canada, Mexico, Central and South America and the Caribbean (Reisen and Brault 2007)

The phylogenetic relationship of WNV strains is broken into two lineages based on amino acid substitutions or deletions in the envelope protein (Brinton 2002). Lineage 1 is associated with all cases of severe disease and it is the most widespread of the lineages including the

introduced strain to the United States (NY99). Lineage 2 is restricted to Africa, and has never been the cause of severe disease or death, but is associated with WN fever (Brinton 2002). Serologically, WNV is most closely related to flaviviruses in the Japanese encephalitis complex, which includes Japanese encephalitis virus, Murray Valley encephalitis, Alfuy virus, and St. Louis encephalitis virus (SLEV) (Brinton 2002). A subtype of WNV, Kunjin virus, is found in Australia and Southeast Asia (Hayes et al. 2005).

Transmission Cycle

West Nile virus is an arthropod-borne virus (arbovirus) with a natural transmission cycle between mosquito vectors and wild birds that serve as amplification hosts. Mosquitoes in the genus *Culex* have been widely implicated as primary vectors of WNV (Andreadis et al. 2001, Hayes 1988, Nasci et al. 2001b, Trock et al. 2001, Turell et al. 2001). *Culex univittatus* Theobald is considered the primary vector in Africa and *Culex pipiens* L in Europe (Hubalek and Halouzka 1999). In Asia *Cx. vishnui* is the primary vector. The primary North American vectors of WNV are *Culex* spp. with great regional variation. In the northeast, *Cx. pipiens* is the primary vector; in the southeast *Cx. quinquefasciatus* is considered an important vector, and in the west, *Cx. tarsalis* appears to be the primary vector (Sardelis et al. 2001, Goddard et al. 2002, Kilpatrick et al. 2005). Other species may be important depending on geographic location and environmental conditions (Kilpatrick et al. 2005). In Florida, *Cx. nigripalpus* is a primary vector for WNV (Rutledge et al. 2003).

West Nile virus is zoonotic and is maintained in complex life cycles involving birds as the primary vertebrate amplification host and mosquitoes as the principle arthropod vector. The transmission cycle of WNV does not affect humans or domestic animals until the virus escapes its transmission/amplification focus via either the amplifying vector or other mosquito with epidemic potential (Campbell et al. 2002). Humans and domestic animals can develop clinical

illness but are considered dead end hosts because they do not frequently produce sufficient viremia to infect mosquitoes, and therefore, do not contribute to the transmission cycle (Hayes et al. 2005). A transient viremia was documented in horses experimentally infected in Egypt (Schmidt et al. 1963), and a similar study by Bunning et al. (2002) found low level titers (a maximum viremia of $10^{3.0}$ PFU/mL) which were insufficient to infect *Aedes albopictus*. High titers were found in the brain and spinal cord but not in the blood (Bunning et al. 2002). Even if this low viremia results in transmission to a mosquito, a lower infective titer is correlated with a reduced transmission rate overall (Bunning et al. 2002).

Humans and horses are susceptible to infection when the virus has become amplified throughout the resident avifauna. Amplification involves a cascade of virus transmission between infected birds and competent mosquito vectors. If the proper environmental conditions persis, this bird to mosquito to bird amplification cycle can result in a large number of infective mosquitoes. After a period of efficient virus amplification, often late in the summer, there is transmission to the human and horse population (Petersen et al. 2003). Direct human-to-human transmission does not occur, although direct transmission has been documented for birds (Austin et al. 2004, Banet-Noach et al. 2003), and farmed alligators (Jacobson et al. 2005). Horizontal transmission (non-vector) in humans is possible through breast milk, blood donation, trans-placental transmission, and organ transplant (Hayes and O'Leary 2004).

An understanding of arboviral transmission cycles begins with the correct identification of biologically significant vectors. West Nile virus has been isolated from 62 mosquito species collected in North America {2007 1905 /id}. It is unlikely that many of these mosquito species play a significant role in the transmission of WNV (Hayes et al. 2005). In order to be implicated as a vector, a mosquito must fit the following four criteria. 1) The mosquito must be

physiologically able to replicate the virus and to infect a naïve host (vector competence). 2) The mosquito must survive the extrinsic incubation period, which is the time necessary for the virus to replicate in the mosquito (10 to 20 days for WNV). 3) The mosquito must feed on a susceptible host. And 4) in order to be a bridge vector (one taking the virus from the mosquito-bird cycle and transmitting to secondary hosts) it must be an indiscriminate feeder. There has been laboratory confirmation of vector competence of several species (Turell et al. 2005). In Florida, there are 80 mosquito species (Darsie and Morris 2003), and very few meet the criteria of a vector outlined above.

West Nile virus undergoes four phases in the yearly cycle of transmission: maintenance, amplification, early transmission, and late transmission (Shaman et al. 2003). Several abiotic factors or non-living components of the environment are considered determinants for viral levels seen in host and vector populations each year. In peninsular Florida, the maintenance phase is from January to March, the amplification phase is from April to June, early transmission is from July to September, and late transmission is from October to December (Shaman et al. 2003). During the maintenance phase the virus survives the peninsular Florida dry season. It is not clear where the virus is during this phase, but low level transmission between mosquito vectors and susceptible wild birds is probable. Virus may be maintained throughout the winter in overwintering mosquitoes, chronic infection in birds, and by continued enzootic transmission (Reisen and Brault 2007). Drought conditions limit the available water and bring mosquito populations into contact with susceptible wild birds, thus facilitating WNV amplification. Once rainfall increases, infected mosquitoes are able to disperse into new habitats. Infected female mosquitoes then transmit WNV when feeding on a susceptible host. During years when drought

brings mosquito and bird populations into close proximity the amplification phase can be quite intense and early transmission is more often seen (Shaman et al. 2005).

Clinical Disease

Human

The most clinically susceptible hosts of WNV appear to be humans, horses and corvids. The symptoms of disease in humans range from sub-clinical, to encephalitis, coma, or even death (Petersen and Marfin 2002). In 80% of the cases WNV infection is sub-clinical (Mostashari et al. 2001). The remaining 20% of infected people will develop symptoms ranging from mild (headache, body ache, and flu-like symptoms) to severe (severe headache, stiff neck, convulsions, coma, and death) (Bernard and Kramer 2001, Petersen and Roehrig 2001). The incubation period (time from infection to onset of systems) lasts about 3 to 14 days with symptoms lasting between a few days and a few weeks (Jeha et al. 2003, Mackenzie et al. 2004)

West Nile fever refers to less severe cases that are self-limited and often resolve within a week. West Nile encephalitis and West Nile meningitis are more severe forms of the disease that affect the nervous system and may persist for over a month (Hayes et al. 2005). Encephalitis refers to an inflammation of the brain and meningitis is an inflammation of the membrane around the brain and the spinal cord (Mostashari et al. 2001). The people at highest risk of severe disease outcome are those over 50 years of age, or that are immune compromised. Less than 1% of all infected persons will develop severe disease (Hayes et al. 2005).

Equine

Clinical symptoms in horses and other equids (ponies, donkeys, mules) range from asymptomatic to fatal (Ostlund et al. 2001, Porter et al. 2003). Approximately 10% of infected horses and other equids develop clinical symptoms. The clinical signs in horses are most commonly ataxia, weakness, and changes in mental state (Cantile et al. 2000). Early reports

during the U.S. WNV outbreak predicted an incubation period from 3 to 14 days and symptoms lasting between a few days to a few weeks (Ostlund et al. 2001). In experimental inoculations, horses become viremic between 2 and 5 days after infection and develop clinical disease between 9 and 14 days post inoculation, which is consistent in all methods of infection including needle, mosquito and intrathecal routes. The outcome is fatal in 35 to 45% of clinically affected horses (Bunning et al. 2002, Long et al. 2007). In New York a seropositive rate of 29% was documented when asymptomatic stable mates of confirmed horses were tested (Trock et al. 2001). The increased rate of seroprevalence was likely an indication of the increased arboviral activity in the area.

Avian

Symptoms in birds infected with WNV may range from asymptomatic to fatal (Komar, 2003). Avian mortality in the Old World was relatively uncommon prior to the introduction of WNV to North America in 1999. The neurological invasion of WNV in domestic geese (1997), and in a flock of storks (1998) in Israel, are among the few reports of WNV causing death in birds in the Old World (Malkinson et al. 2002, McLean et al. 2002). In North America there have been 198 species of birds reported to be susceptible to a fatal outcome when infected with WNV (Komar et al. 2003). Corvids (Passiformes) are especially susceptible to infection. Signs of infection include lethargy, recumbency, and hemorrhage (Komar 2003). During epizootics, (outbreaks in the bird population) there is a high rate of natural infection in birds (Work et al. 1955, McIntosh and Jupp 1982, Malkinson and Banet 2002, Komar 2003). Multiple tissues are damaged with infection and the cause of death is likely multiple organ failure (Komar 2003).

Other Vertabrates

Experimental infections of WNV in other domestic animals have shown that development of viral titer and clinical signs are relatively rare (Blackburn et al. 1989, McLean et al. 2002.

Sheep that were fed on by WNV infected mosquitoes did not mount a viremia (McLean et al. 2002). In a seroprevelence study, in eastern Slovakia, WNV antibodies were detected in 1% of 608 sheep screened (Hubalek and Halouzka 1999, McLean et al. 2002). Calves that were experimentally infected did not produce viremia. In a seroprevalence study in Romania, 4.9% of sheep, 4.1% of cattle, and 12% of goats had HI antibody for WNV (Hubalek and Halouzka 1999, McLean et al. 2002, Murgue et al. 2002). Dogs that were inoculated subcutaneously with WNV developed antibody titers and one dog developed a low titer viremia (Blackburn et al. 1989). A survey of dogs in South Africa found 46% of 377 dogs screened had HI antibodies against WNV (Blackburn, 1989, McLean et al. 2002). A water buffalo fed on by infective WNV mosquitoes did not produce detectable viremia, and in a seroprevalence study 72% of water buffalo sampled had neutralizing WNV antibody (McLean et al. 2002). Farmed alligators are susceptible to fatal infection in North America (Jacobson et al. 2005) and develop extremely high viral loads in the blood. Lake frogs in Russia are apparently competent reservoirs for WNV (Hubalek and Halouzka 1999, McLean et al. 2002).

Epidemiology and Ecology

The numbers of human cases, horse cases, and positive surveillance reports were highest in Florida between 2001 and 2003, and since that time have decreased. The pattern of WNV dispersal in the United States has usually displayed a three-year cycle (Reisen and Brault 2007). The entry year is often followed by transmission at epidemic levels (in Florida the highest levels occurred two years after WNV was first reported in 2003 with 82 human cases). A decrease in WNV activity is observed after a large epidemic occurs in a new geographic setting (Reisen and Brault 2007). Interestingly, as WNV becomes established in a new geographic focus, reports of SLEV activity in the area decline (Reisen and Brault 2007), although this was not the case in Florida where sentinel chicken seroconversions to SLEV > WNV in 2006. The decline of SLEV

in many areas is most likely due to the partial cross protection against WNV provided by previous exposure to SLEV in both birds and mammals (Tesh et al. 2002, Fang and Reisen 2006). West Nile virus epidemics (just as SLEV epidemics) require a number of complex ecological factors to be in place. West Nile virus may also be subject to epidemiological conditions such as local bird population susceptibility, rainfall patterns, and mosquito vector dynamics for transmission to occur.

Invertebrate Hosts (vector)

The enzootic (within animal) WNV transmission cycle includes an avian reservoir (amplification host) and a mosquito vector. After feeding on an infectious blood meal, WN virions enter the mosquito midgut and infection of the midgut epithelium may follow (Brinton 2002). In a competent vector (an arthropod capable of becoming infective), the virus replicates in the cells of the midgut epithelium and subsequently is released into the body of the mosquito resulting in a disseminated infection (Scholle et al. 2004). Virus then enters other organs, including the salivary glands, via the hemolymph. After replication in the salivary glands transmission to a host by probing or taking a subsequent blood meal can occur (Scholle et al. 2004). Differences in both midgut and salivary gland infection and escape barriers may explain variations in mosquito vector competence.

Additional biological routes of infection include transovarial (entry of virus into mosquito eggs during oviposition) and venereal (female to male) transmission. Mid-winter isolations of WNV from overwintering *Culex* mosquitoes demonstrates the potential of the virus to persist until spring and emerge with mosquitoes to reestablish an enzootic transmission cycle in the area (Nasci et al. 2001a). Vertical transmission may contribute to maintenance of WNV (Miller et al. 2000). Mid-winter isolations of WNV are most likely from a mosquito undergoing diapause (hibernation physiology and behavior) and because normally the female does not first blood feed,

it can be reasonably assumed a mid-winter infection of WNV was acquired transovarially (Komar 2003). Alternatively, *Culex* infected by feeding on a viremic vertebrate host may have survived the winter. Transovarial transmission of WNV and preservation of the virus in hibernating mosquitoes are not thought to play an important role in the maintenance of the virus in nature, but the potential of alternative routes of transmission such as vertical transmission do exist.

The most important mosquito genus in terms of WNV transmission is *Culex*. The majority of WNV field isolations have been from *Culex* mosquitoes, and in field studies, *Culex* spp. have in repeated investigations, the highest minimum infection rates (MIR) relative to other mosquito species (Nasci et al. 2002, Kilpatrick et al. 2005). Because of the midgut barrier, *Culex* mosquitoes do not have the highest vectoral capacity (physiological ability to transmit the virus) as compared to container breeding *Aedes* spp. and *Ochleratatus* spp. Despite a lower vectoral capacity, other factors such as mosquito density, biting preference and seasonal activity makes *Culex* species the most important mosquito genus in WNV transmission (Nasci et al. 2002, Kilpatrick et al. 2005). Finally, each mosquito species may demonstrate a range in vectoral capacity because ambient temperature, infective dose (from blood), and length of extrinsic incubation period influence the efficiency of a vector under field conditions.

Several *Culex* species are involved in the transmission cycle of WNV and preferentially feed on birds thereby amplifying the virus in avian populations. Ornithiphilic (avian-feeding) species such as *Culex nigripalpus, Culex pipiens, Culex quinquefasciatus,* and *Culex tarsalis* are considered maintenance and amplification vectors of WNV (Turell et al. 2005). Host-shifting behavior (a host preference switch from birds in the spring to mammals in the fall) seen in *Cx. nigripalpus, Cx. tarsalis,* and *Cx. quinquefasciatus* may drive WNV transmission to human and

horse populations late in summer and early fall by brining the virus from its point of focal transmission out to exposed hosts (Kilpatrick et al. 2005). This occurs when mosquitoes first feed on an avian host and become infected. After completing the extrinsic incubation period the mosquito may transfer the virus at a subsequent blood meal by probing a susceptible mammalian host.

An infective mosquito can deliver approximately $10^{4.3}$ plaque forming units (PFU)/mL of WN virus to a host, with a range of viral titer (amount of virus in the salivary glands) among individual mosquitoes and species (Vanlandingham et al. 2004). *Aedes albopictus* is a competent vector of WNV. *Aedes albopictus* experimentally infected with WNV developed titers between $10^{6.6}$ to $10^{7.9}$ PFU per mosquito. When subsequently fed on horses, this titer was sufficient to infect the majority of horses, which developed a low-level viremia ranging from $10^{1.0}$ - $10^{2.7}$ PFU/mL (Bunning et al. 2002). None of the horses (n = 12) were able to re-infect mosquitoes. The minimum host viremia capable of infecting a mosquito vector varies by mosquito species, but the relationship between susceptibility to WNV infection is dose dependent and approaches 0 below $10^{4.0}$ PFU/mL (Reisen et al. 2005). Serum titers below $10^{4.3}$ PFU/mL are not capable of infecting most mosquito species when feeding on rabbits with low-level viremia (Tiawsirisup et. al 2005). *Culex tarsalis* is considered one of the most efficient vectors and when fed on a blood meal containing $10^{4.9}$ PFU/mL the number of mosquitoes that became infected ranged between 0%-36% (Hayes et al. 2005). Concentrations of $10^{7.1}$ PFU/mL are required before 74%-100% of *Cx. tarsalis* become infected when fed on an infectious blood meal (Hayes et al. 2005). The threshold necessary to infect *Cx. pipienes* and *Cx. quinquefasciatus* is $10^{5.0}$ PFU/mL (Allison et al. 2004). It is possible that hosts that maintain a low viremia (below the threshold) for an extended amount of time may encounter many

mosquitoes and successfully infect a small number of them (Lord et al. 2006). These instances could be considered as secondary routes of transmission in the WNV cycle.

Vertebrate Hosts (Reservoir/Amplification Host)

The most important amplification hosts of WNV are avian. Laboratory studies have shown that member of the orders Passeriformes (song birds), Charadriiformes (shorebirds), Strigiformes (owls), and Flaconiformes (hawks) develop blood virus levels sufficient to infect most feeding mosquitoes (Komar 2003, Komar et al. 2003). Passerines, such as common grackles (*Quiscalus quiscula*), house finches (*Carpodacus mexicanus*), and house sparrows (*Passer domesticus*) are capable of infecting many mosquitoes (Komar 2003). Serosurveys have demonstrated that house sparrows are frequently infected with WNV (up to 60%), may develop a high viral titer of sufficient duration and magnitude to infect vector mosquitoes, and are abundant (Komar et al. 2001, Komar et al. 2003, Godsey et al. 2005). These attributes allow house sparrows to potentially serve as important amplifying hosts. Crows may experience up to 100% mortality in some outbreaks (Komar et al. 2001) and their rapid fatality likely limits their reservoir potential. Some resident birds were found to have seroprevelence rates of 20 to 50% in the epicenter of WNV outbreaks (Komar et al. 2001) which in migratory birds the seroprevelence was 0.8% (McLean et al. 2002). The importance of migratory birds in dispersing WNV remains uncertain, but it has been suggested that movement of resident birds, nonmigratory birds, and migratory birds may contribute to the spread of WNV (Reed et al. 2003, Petersen et al. 2003).

Field observations of direct bird-to-bird transmission have not been made, but laboratory tests confirm this probability. Infected birds caged with uninfected birds are able to spread WNV (McLean et al. 2002); the mode of transmission is likely low-level viral shedding per os (oral) or per cloaca (cloacal). Oral transmission in crows and geese has been documented by

ingestion of infected water, mosquitoes, or carrion (Langevin et al. 2001, McLean et al. 2002, Banet-Noach et al. 2003).

Although WNV has been isolated from some mammals, and there have been occasional reports of mammals spiking sufficient viremia to infect mosquitoes, in general mammals are commonly considered dead-end hosts because they do not usually spike a sufficient viremia to infect a feeding mosquito and thereby do not contribute to the continuation of the virus cycle (Hayes 1988, Bunning et al. 2002). Rabbits were found to be capable of infecting various mosquito species and developing a short-lived viremia of up to $10^{5.8}$ PFU/mL (Tiawsirisup et al. 2005). Other mammals experimentally infected such as horses, cats, dogs, and mice rarely exhibit titers above $10^{4.0}$ PFU/mL whereas birds such as passerines can exceed $10^{6.0}$ PFU/mL for a few days (Tiawsirisup et al. 2005). Corvids are the most susceptible to infection producing high viremia of over $10^{10.0}$ PFU/mL (Reisen et al. 2005). The corvids usually are moribund (approaching death) after 5-6 days postinoculation. Blood virus levels in naturally infected rock pigeons ranged from $10^{2.3}$ PFU/mL – $10^{7.2}$ PFU/mL (Allison et al. 2004). Chickens remain valuable in sentinel programs because, even though chicks can develop a substantial viremia, the average adult viremia is $<10^{4.0}$ PFU/mL, which is insufficient to infect most mosquitoes (Langevin et al. 2001).

Human

In humans, patients develop an average viremia of 0.1 PFU/mL (ranging from 0.06-0.50 PFU/mL) (Montgomery et al. 2006). Blood screened from donors in the US in 2002 had a maximum titer of $10^{3.2}$ PFU/mL (Hayes et al. 2005). This level is safely below the viremia required to infect most efficient vectors. Therefore, humans do not likely contribute to the WNV transmission cycle and can be considered dead-end hosts. Despite findings that some children in

Israel spiked viremias sufficient to infect mosquitoes, humans are still considered dead-end hosts (Hayes and O'Leary 2004).

Seroprevelence of WNV in a 1999 New York study (Mostashari et al. 2001) was 1 in 150 infections resulted in meningitis or encephalitis. A 2000 study in New York again found a similar result (CDC 2001). The results of the two serosurveys were consistent with a previous study in Romania (1996) indicating that 1 in 140 to 320 infections led to these clinical outcomes (Tsai et al. 1998). The case fatality rate in the US in 2002 for human cases of WNV with meningitis was 2%, and the case fatality rate for those with encephalitis was 12% (O'Leary et al. 2004).

Surveillance and Detection of West Nile Virus

Florida has had an arthropod borne virus (arbovirus) surveillance program in place since 1977 to track the amplification and transmission of mosquito-borne viruses including eastern equine encephalitis virus (EEEV) and St Louis encephalitis virus (SLEV) (Day and Stark 1996). The Florida state department of health (DOH), division of environmental health, coordinates the surveillance program. The Interagency Arbovirus Surveillance Network reports to the DOH and is composed of several local, state and federal agencies, which are involved with the surveillance and control of arboviral diseases.

Upon its arrival in the United States, WNV was easily added to the existing surveillance program with the addition of WNV-specific laboratory diagnostics. Because SLEV and WNV are antigenically related, cross-reactions are observed with some serologic tests and so plaque reduction neutralization testing (PRNT) is done to distinguish the two viruses. Due to the correlation of WNV-positive dead bird reporting and local WNV transmission, dead bird reporting has become a valuable surveillance tool in the United States (Eidson et al. 2001a, Eidson et al. 2001b, Nasci et al. 2002).

Horses have been found positive for IgM antibody 8 to 10 days after infection with WNV. The IgM antibodies may persist for 2 to 3 months, most horses only have antibody for 3-4 weeks which makes this test ideal for detection of recent infection. West Nile virus neutralizing antibodies can persist for years after infection (Durand et al. 2002). Horses are not currently used as part of an active WNV surveillance program in the United States, but data is collected passively on all confirmed horse cases in the U.S. by the CDC. In New York State horse positives were unreliable in the prediction of human cases of WNV (Trock et al. 2001). It is not yet known whether WNV surveillance in horses can predict human cases in Florida, but horse cases that are reported to local health departments are used as part of arbovirus surveillance. Blackmore et al. (2003) reported that the epicenter of the 2001 WNV outbreak in Florida horses was in Jefferson County. From Jefferson County, the outbreak spread east, west, and south to a total of 40 Florida counties with confirmed horse cases. In the counties reporting both horse and human cases, the horse cases preceded the human cases by one to four weeks (Blackmore et al. 2003). Because horse cases generally precede human arboviral infections, local identification of horse cases is an important part of the passive surveillance network in Florida and the U.S.

Diagnostics

Routine arbovirus surveillance methods include screening of mosquito pools by viral isolation, or by antigen detection. Viral isolation is typically carried out in the cell line C6/36 (*Aedes albopictus)* or in Vero cells. Cell culture procedures detect live virus in the sample. Enzyme Linked Immunosorbent Assay (ELISA) can detect viral antigen in mosquito pools, avian tissues, and human tissues. Frequently, viral nucleic acid detection methods such as real time quantitative RT-PCR are used by local health departments for screening of mosquito pools {Stark, 2006 20 /id}. The VecTest™ (Medical Analysis Systems, Camarillo, CA) is a rapid immunochromatographic test developed for the detection of viral antigen. The VecTest can be

used directly in a mosquito pool homogenate, eliminating the need for lengthy laboratory preparation.

Vero cell culture and RT-PCR were used to confirm the index case (initial case identified in an outbreak) in the United States (Huang et al. 2002). When cell culture is used for WNV isolation, it is usually in conjunction with RT-PCR because the latter is more sensitive. Using RT-PCR, one infected mosquito can be detected in a pool of 50 mosquitoes (a standard procedure for surveillance of mosquito populations) because the limit of detection in RT-PCR is 40 RNA copies (Shi et al. 2001). This sensitivity is more than adequate for WNV screening since a mosquito capable of transmitting virus contains more than 10^5 PFU of virus (Hadfield et al. 2001). Lanciotti and Kerst (2001) found that nucleic acid amplification assays were far more sensitive for screening mosquito pools than relying on cell culture alone. The use of RT-PCR increased the detection of virus and significantly decreased the amount diagnostic laboratory time (Lanciotti et al. 2000, Lanciotti and Kerst 2001).

Diagnostic methods for WNV detection in horses changed after the introduction of a vaccine. In 1999, the primary diagnostic tool was the plaque-reduction neutralization test (PRNT) of equine serum for confirmation of WNV infection and virus isolation from equine brain or spinal cord tissue (Ostlund et al. 2001). To update the diagnostic tools available the (Ig)M-capture enzyme-linked immunosorbent assay (MAC-ELISA) was developed (Ostlund et al. 2001). The assay was modeled after the EEEV MAC-ELISA. Upon experimental challenge in horses, Immunoglobulin (Ig) M isotype anti-WNV antibodies become detectable 8-10 days after infection and persist up to two months (Ostlund et al. 2001). This test has a sensitivity and specificity of 91.2% and 99.7%, respectively, for confirming recent infection in equids with

encephalitis (Long et al. 2006). West Nile virus neutralizing antibodies may be detectable in horse sera for years after infection (Durand et al. 2002).

Neutralizing antibodies to WNV may persist for more than two years following infection. Neutralizing antibodies can also be passed from mare to foal via the colostrum (milk). Due to the properties of neutralizing antibodies, the MAC-ELISA is an important diagnostic tool to identify recently infected horses in areas where previous infection has occurred because the IgM antibody response wanes more rapidly than neutralizing antibodies to WNV. Additionally, the MAC-ELISA is capable of producing reliable results even in vaccinated horses (Porter et al. 2003). The ELISA detects antibodies to WNV and can indicate if the horse had been exposed even without clinical symptoms.

Risk Factors

Because arboviruses are maintained in complex cycles of avian hosts (reservoirs) and mosquito vectors, a number of factors must be in place for epidemic transmission to occur. Abiotic factors greatly affect the year-to-year transmission patterns observed by facilitating the interactions of infective mosquitoes and susceptible hosts. Weather influences WNV transmission by affecting the distribution and abundance of mosquito vectors and the time of extrinsic incubation period (Reiter 1988). There is abundant research on the predictive factors to human arboviral outbreaks and the most reliable indicators are rainfall patterns, sentinel chicken conversions, and a large juvenile bird population (Ruiz et al. 2004, Day and Lewis 1991). The single most important risk factor to human transmission is an abundant infective mosquito population (Komar 2003).

Prediction of Human Cases

The principle reason for the active surveillance of arboviruses is to protect the public. Various surveillance tools including mosquito collection, dead bird testing, sentinel chickens,

and horse cases (Blackmore et al. 2003) are used to monitor arboviral amplification and transmission. Together these surveillance techniques are used to make public health decisions, by comparing arboviral activity to baseline historical data, in order to protect the public against an outbreak of a particular arbovirus (FDOH 2007). A public health advisory (by radio, television and print) can be released that advises people to stay indoors during hours of heavy mosquito activity, reduce exposure to mosquitoes, and take preventative measures. Preventative measures include wearing protective clothing, and using chemical repellants. When necessary, public health measures can be implemented as was done in Florida during the 1990 outbreak caused by SLEV (Day 2001). All factors including human cases are part of the assessment risk resulting in a health advisory. Reliance only on human disease results in dissemination of information after an epidemic is often well underway. Reliable prediction assists policy and regulatory officials focus control efforts that reduce the possibility of human cases before any disease occurs. In Florida, animal, mosquito, and chicken seroconversion data is compiled weekly and released by the Florida Department of Health. In addition representatives from the Arbovirus Interagency Task Force discusses additional options for media release and public health advisories relating to the weekly data.

Blood Meal Analysis

Knowledge of mosquito host feeding patterns provides insight to viral transmission cycles through investigations of the role of a vector mosquito in enzootic transmission among avian hosts or epidemic transmission outside of this cycle to mammalian hosts. Techniques in blood meal analysis have been changing since the early 1920s and have included direct observation of feeding mosquitoes, host-baited trap catches, and serological and genetic based techniques (Ngo and Kramer 2003). The most common serological and genetic based techniques have been the precipitin test, the Enzyme Linked ImmunoSorbent Assay (ELISA) and Polymerase Chain

Reaction (PCR) assays (Ngo and Kramer 2003). Polymerase Chain Reaction amplification of host DNA followed by sequencing is becoming a common method for blood meal detection and has several advantages over precipitin tests and ELISA. Due to the sensitivity of the PCR, a very small amount of DNA can be used as template so even partially engorged mosquitoes can yield a blood meal confirmation. Additionally, with serological tests such as precipitin and ELISA, anti-sera must be prepared for each potential host species allowing blood meal confirmation to only a limited number of species. With the advent of web-based databases such as GenBank, it is now possible to compare nucleotide sequences and determine the exact identity arthropod blood meals.

In precipitin tests, the blood meal suspension is mixed with the antiserum of various vertebrates and if a reaction is observed then a precipitate will form and the blood meal is considered positive for that host type (Tempelis 1989). The ELISA uses a species-specific antibody that will react with the blood meal and result in a color change signally that binding of the antibody has occurred. These techniques can identify blood meals to general groups of animals such as avian vs. mammal and within mammalian hosts, human, cow, horse, etc. Genetic methods allow for species-specific identification for birds to the species level. Using restriction fragment length polymorphism (RFLP) analysis, Kirstein and Gray (1996) were able to identify genera level mammalian blood meals from *Ixodidus* ticks. Heteroduplex analysis (HDA) helped classify mosquito-feeding patterns in the Tennessee valley area to the species level (Lee et al. 2002a). Sequencing blood meal polymerase chain reaction (PCR) product is another way of determining to the source of the blood meal to the species level. The most common technique today relies on the genetic characterization of the blood meal to determine the host. Typically primers in the cytochrome b genome are used to amplify a vertebrate-specific

region from the blood meal DNA. The product of the PCR is sequenced and then matched with known published sequences in the BLAST database of GenBank (NCBI 2008). Host DNA can be detected for up to 72 hours after the mosquito takes the blood meal (Ngo and Kramer 2003).

Many blood meal analysis studies focus on avian hosts for purposes of understanding the commonly fed upon reservoirs in the WNV transmission cycle (Lee et al. 2002a, Ngo and Kramer 2003). Others have done work on avian and mammalian hosts (Apperson et al. 2002, Molaei et al. 2006). Cupp et al. (2004) studied the potential role of reptiles in the WNV transmission cycle by analyzing blood fed mosquitoes that fed on reptilian hosts.

The collection of blood fed mosquitoes is often done by the method of vacuum aspiration and the use of resting boxes (Edman 1971, Edman 1979). Baited CDC light traps generally attract host seeking mosquitoes, but may collect females that are partially engorged, fully engorged, and gravid. Aspiration collections are made in vegetation, natural or man-made structures, and in likely resting habitats such as around tree roots or from resting boxes. The vacuum sucks the mosquitoes into a collection container with a screen to hold them in until they are transferred to another container (Holck and Meek 1991). In Florida, vacuum aspiration has been used to collect mosquitoes in the genera *Culex, Aedes, Anopheles, Coquillettidia, Mansonia,* and *Psorophora* (Niebylski et al. 1994) Day and Curtis 1993, (Edman 1971, Edman 1979). The CDC light traps generally use white light, although alternate colors may be used to increase catch and the traps are frequently baited with CO_2 to attract host seeking mosquitoes (Service 1976). As mosquitoes approach the trap, a fan-generated air current pulls them into a collection bag (Sudia and Chamberlain 1988). Incandescent lights have been shown to be attractive to *Uranotaenia sapphirina* (Osten Saken), *Anopheles crucians* (Wiedemann), *Aedes vexans* (Meigen), *Anopheles quadrimaculatus* Say, *Cx. nigripalpus*, and *Culex* in the subgenus

Melanoconion (Love and Smith 1957, Burkett et al. 1998). To maximize catch, the optimal time to run the trap is coincident with maximum flight activity, the crepuscular period (dusk and dawn) (Bidlingmayer 1967).

Resting boxes are designed to mimic a natural resting habitat (Edman et al. 1968). They often attract blood-engorged females seeking a dark resting place to digest the blood meal. Mosquitoes most often enter in the morning and may leave during the day as the temperatures rise (Edman et al. 1968).

Many critical questions regarding risk factors of WNV transmission to horses exist. Although an efficacious vaccine is available, horse cases continue to occur annually throughout the nation. The study of extrinsic risk factors to horses will help horse owners better understand the environmental risk factors and farm management practices associated with an increased risk of WNV transmission. This knowledge can help broaden our understanding of the epidemiology and ecology of WNV in Florida. Additionally, a full understanding of vector host interactions is still incomplete. The work presented here helps identify the mosquito species feeding on horses. Knowledge of blood feeding habits can be used along with vector competence studies and mosquito life history studies, to incriminate potential mosquito vectors of WNV to horses in Florida.

CHAPTER 2
EXTRINSIC RISK FACTORS ASSOCIATED WITH WEST NILE VIRUS INFECTION IN FLORIDA HORSES

Since its introduction to the United States in 1999, West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) has been a growing public health concern. West Nile virus is a zoonotic (naturally transmitted between vertebrate animals and humans) arthropod-borne virus (arbovirus). West Nile virus is maintained in a complex life cycle involving a primary vertebrate host (passerine birds) and a primary arthropod vector (*Culex* mosquitoes). Susceptible wild birds and vector mosquitoes amplify WNV in foci where mosquito and bird populations are sympatric. *Culex* mosquitoes have been widely implicated as the primary vector of WNV (Andreadis et al. 2001, Hayes 1988, Nasci et al. 2001b, Trock et al. 2001, Turell et al. 2001). The natural cycle of WNV does not affect humans or domestic animals unless the virus escapes its amplification focus. This occurs when infective mosquitoes disperse from amplification foci and bite a susceptible secondary (horses or humans) host (Campbell et al. 2002, Petersen et al. 2003. Humans and horses can develop clinical illness, but are considered dead end hosts because they do not produce sufficient viremia to infect mosquitoes, and therefore, do not contribute to the amplification cycle by infecting additional vector mosquitoes (Bunning et al. 2002, Hayes et al. 2005). Direct (non-vector) transmission has been documented for birds (Austin et al. 2004, Banet-Noach et al. 2003) and farmed alligators (Jacobson et al. 2005). Direct human-to-human transmission is limited to infection through blood transfusion, breast milk, and organ transplant (Hayes et al. 2005).

In Florida, WNV continues to threaten the health of humans and horses. Between 2001 and 2006, there were a total of 1,082 WNV horse cases reported in the state {2007 1906 /id}. Florida plays an important role in the equine industry; many breeding horses are located within the state and are at risk of infection even with the availability of three commercially licensed

vaccines. Despite the availability of a vaccine protecting horses against infection with eastern equine encephalomyelitis virus (family *Togaviridae*, genus *Alphavirus,* EEEV), horse cases are reported regularly. As WNV becomes established, recurrent epidemics and epizootics will most likely occur (Komar 2003). Although Florida has not yet experienced a major human epidemic of WNV, the endemic and epidemic presence of St. Louis encephalitis (family *Flaviviridae*, genus *Flavivirus*, SLEV), which shares an epidemiology similar to that of WNV, suggests that the necessary ecological variables are present in Florida to support future epidemics caused by WNV.

The annual occurrence of WNV infection in horses corresponds with the cycling of the virus in amplification hosts and mosquito vectors in enzootic habitats {2003 645 /id}. Human and equine cases of WNV peak in Florida in late summer and decline after November. Several extrinsic factors such as rainfall (Shaman et al. 2005), avian population dynamics (Ward et al. 2006), and temperature (Dohm et al. 2002) have been correlated with WNV outbreaks in humans and horses. However, there are few data regarding the extrinsic factors related to the ecology of horse farms and the risks associated with farm management practices and disease manifestation in horses. Retrospective studies have been performed that examine clinical disease associated with infection, treatments, and outcomes {Salazar, 2004 342 /id} {Schuler, 2004 270 /id} Epp et al. 2007).

This study is an analysis of extrinsic risk factors that were collected for horses tested for arboviral infection in Florida between 2001 and 2003. This particular study focuses not only on clinical signs but also on the ecology of horse farms where WNV cases were reported. Given that year round management of horses reflects the subtropical Florida climate, there are likely unique factors that intersect with horse husbandry that create risk for horses. Since WNV is a

reportable disease in humans and horses in Florida, all veterinarians are required to submit an arbovirus case information form (ACF) to the Florida Department of Agriculture and Consumer Services (FDACS). This highly detailed form allowed for the development of a database and the opportunity for a follow-up survey of horse owners.

The primary objective of this research was to identify factors contributing to the total WNV equine cases from 2001 to 2003 in Florida. The factors of interest in this study were farm management, farm ecology, and the entomological conditions associated with each farm. The central hypothesis, that risk factors for WNV transmission in horses are related to the availability of mosquito larval habitat, animal housing conditions, and animal management practices was investigated.

Materials and Methods

Arbovirus Case Information

The arbovirus case information form (ACF) provided specific information about each horse tested including stable location, signalment (clinical signs and symptoms), individual history (age, sex, breed), date of onset of clinical signs, and date of testing. Space was provided on the form to note any other clinical signs and a brief history of clinical presentation of the horse (Appendix A). The FDACS provided copies of the ACF information to the Emerging Diseases and Arbovirus Research and Test Program (EDART) at the University Of Florida College Of Veterinary Medicine in Gainesville, Florida for data entry and analysis for all horses tested in Florida from 2001 to 2005. All data were entered into a database (Microsoft Excel and Access, Microsoft Corporation, Redmond, Washington) and coded for statistical analysis.

Retrospective Survey

Information was taken from the ACF to create a follow-up survey (Appendix B), which was mailed to the owners of all horses that were tested for arboviruses from 2001to 2003. A

50% return of the questionnaire was the targeted response rate. The questionnaire focused on horse husbandry, farm management, and farm ecology (Table 2-1). Respondents were asked to check the most appropriate category in response to each question. A cover letter describing the objectives of the study and a promise of anonymity to participants accompanied the questionnaire (Appendix C). Reminder postcards were sent two and four weeks following the initial mailing. A second mailing was sent to the owners who did not respond to the initial survey within two months of the first mailing. Two reminder postcards were mailed out two and four weeks following the second mailing.

Case Definition

Positive Horses. A confirmed horse case was defined as manifestation of WNV clinical signs (Appendix A) and one or more of the following: isolation of WNV from tissue, blood, or CSF; detection of a positive IgM antibody to WNV by MAC-ELISA in a single serum test, and in the first year (2001) of WNV encroachment, a four-fold rise intheWNV plaque reduction neutralization test (PRNT). After 2001, it was presumed that vaccinated horses would have neutralizing antibody precluding the usefulness of the PRNT. All confirmed positive horse cases in the state of Florida from 2001-2003 (n = 534) were analyzed as the positive group in the study of WNV risk factors associated with the farm environment.

Negative Horses. A WNV-negative horse failed to meet the above criteria for WNV infection based on serological testing and/or post-mortem analysis. All horses that tested negative for WNV in the state of Florida from 2001-2003 (n = 402) were analyzed as the comparison control group in the study of WNV risk factors associated with the farm environment.

Statistical Analysis

Responses to survey questions were categorical and statistical analysis for independence was performed with bivariate analysis (SPSS v 15, Chicago, IL). Fisher's exact test was used on variables containing fewer than five responses in a contingency table cell. A Chi-square (χ^2) test was used for analysis of independence between nominal variables that consisted of two or more categories and contained variables with greater than five responses per contingency table cell. Odds ratios were calculated for the dichotomous variables that were significant with χ^2 statistics or logistic regression ($P < 0.05$) in the survey. A cross-table was used to stratify variables and create a contingency table to compare the relationships between variables. Stratified analysis was used to compare WNV test outcomes with the time since last vaccination and the frequency of vaccination separately. A logistic regression analysis was performed using WNV disease status as the outcome (dependent) variable. The data that were included in the regression analysis were from the combined results of the survey and the ACF. Observational (independent) variables tested included sex, vaccination status (those positively indicated on the ACF, all unknowns were treated as missing data points), and each environmental variable.

The logistic regression analysis presented here was adjusted by controlling for vaccination status to clearly identify the risk/protective effects of the environmental variables associated with the individual farm (Table 2-5). These data were not as powerful when separated by year since the separation reduced the sample size and subsequently increased the standard deviation. For all the variables examined (arbovirus prevention, stable characteristics, and farm ecology) the years were combined to keep sample size robust.

All statistical analyses were performed with commercially available software (Minitab v 14, State College, Pennsylvania; EPI-Calc v 1.02, Brixton Books, Brixton, UK; SPSS v 15, Chicago, IL). Multivariate analyses were performed for extrinsic factors using cross-tables and

logistic regression models. The presence of clinical WNV symptoms was the dependent variable for each statistical test. Cross-tables were used to compare two contingency tables and stratify the results to compare two variables such as vaccination status and disease outcome.

Results

The Florida Department of Agriculture and Consumer Services (FDACS) compiles information provided by veterinarians in the state of Florida on every horse tested for viral encephalitis. The veterinarians use an arboviral case information form (ACF) at the time of testing a symptomatic horse (Table 2-1). Between 2001 and 2005 there were 2,824 horses that were classified as either WNV diagnosed (WNVD, n = 1,386 (49%)) or WNV negative (WNVN, n = 1,438) based on clinical symptoms and serological testing (Table 2-2). The retrospective mail survey was sent to all owners of horses tested from 2001 to 2003 (n = 2,501) of which 936 (37%) were completed and returned for 534 positive horses and 402 negative horses.

Farm and Sample Submission Information

West Nile virus positive horses were reported in 55 of 67 counties in Florida from 2001 to 2003. Cases began in the summer of each year (2001 to 2003) and peaked in the fall (September) followed by a sharp decline in the winter (Figure 2-1). Each year the cases were seasonal except in 2002, when cases were reported throughout the year.

There was a significant (P = 0.045) difference of age structure of positive horses between 2001 and the other study years (2002 to 2004). In 2001, young and old horses were affected, whereas in 2002-2004, horses between 1 and 5 years of age were most commonly reported as WNV-positive. In 2001, seven (1.2%) WNV-positive horses were < 1 year old, 324 (56%) were between 1 and 5 years old, 99 (17.8%) were between 5-10 years old, and 145 (25%) were greater than 10 years of age. From 2002 to 2004, 36 horses (9.6%) were < 1 years old, 135 (35%) were

between 1 and 5 years old, 181 (47.3%) were between 5-10 years old, and 31 (8.1%) were greater than 10 years old (Table 2-3).

Quarter horses were the most common breed tested in both the WNVD and the WNVN groups, and there were no significant differences in breed representation in either group. The frequency of WNV testing in most other breeds was similar between groups. There were 626 (45%) females, 680 (48%) geldings, and 93 (7%) stallions that were WNVD. This sex distribution was significantly different ($P < 0.001$) from the WNVN horses where 510 (47%) females, 388 (35%) geldings, and 200 (18%) stallions were tested (Table 2-4). The male to female ratio was about equal in both groups, but significantly more geldings were diagnosed positive for WNV.

Arbovirus Infection Prevention

The prevention of WNV infection in Florida horses included vaccination, use of insect repellents, and barrier protection with fly sheets (a protective covering secured to the horse) (Table 2-1). Insecticides containing permethrin were the most commonly used products for the protection of horses from biting arthropods. There was no statistical difference between the WMVD and WNVN groups regarding the use of permethrin products. Frequency of use of all insect repellents (spray or lotion) was similar between the two analyzed groups. Fly sheets were not frequently used in either group.

Eighty percent of the tested horses had a known vaccination history. Between 2001 and 2003, 1,368 horses were WNVD and 1,184 were WNVN. Forty two percent of all tested horses received a vaccine for WNV prior to the onset of illness. To obtain more detailed information from the owners they were asked if the horse was vaccinated and how many times the horse had been vaccinated each year.

In 2001 there was an increased association of WNVD horses with vaccination (Table 2-5). In 2002 and 2003 the vaccine showed a protective effect. The data were stratified by the number of times the horse was vaccinated and by the time since last vaccination (within 2 weeks, under 6 months, between 6-12 months, or >12 months) in order to more closely examine the relationship between vaccination and disease. Eighty-five (9.6%) of the WNVD horses were vaccinated two weeks prior to the onset of illness, 297 (34%) were vaccinated between two weeks and 6 months, 47 (5.3%) were vaccinated between 6-12 mo, and 16 (1.8%) were vaccinated >12 months prior to onset of illness. Horses were not protected from infection if the vaccine was received within two weeks of WN disease onset or if the vaccine was received over one year prior to WN disease onset. For the group of horses that were vaccinated in the time frame of more than two weeks to under one year prior to the onset of WN disease, the vaccine did decrease the incidence of WNV (Table 2-5).

Owners were asked to indicate the number of times each horse received a WNV vaccine and were given a choice of one to four times. The average response for the combined years of 2001 through 2003 was once (25%), twice (55%), three times (15%), and 4 times (4%). Horses that received only one dose of vaccine did not have protection to WNV. A protective association was seen for horses that were given two or more doses of vaccine (Table 2-5).

Because the timing of vaccine administration was closely associated with protection against WNV infection, vaccination data were sorted into two groups: effective and non-effective vaccine doses received. The effective group was considered to be the group of horses that received the vaccine more than two weeks and less than six months before the onset of illness. When the group of horses that received the vaccine in this time frame was compared to their vaccination status, a strong protective effect of the vaccine was seen (Table 2-5).

Stable Characteristics and Farm Ecology

The construction material of a stable was significantly (P =0.045) associated with WNV infection. For the WNVD group, stables were made of solid wood and cement for 158 (29%) horses, boards with openings for 146 (27%), and an open shed for 73 (13%). The WNVN group had 131 (33%) stables made of solid wood and cement, 79 (20%) made of boards with openings, and 60 (15%) in an open shed (Table 2-6). The logistic model did not indicate a significant correlation with type of stable material and incidence of WNV infection. There was no significant difference if the horse was kept in a pasture versus a paddock. Additionally no significant difference of WN disease incidence was seen for the frequency of stall cleaning.

The presence of fans in a stall (P = 0.04) and the frequency of fan use (P = 0.05) were significantly correlated with WN disease incidence in horses. In the WNVD group 184 (34%) stables had fans, 70 (13%) used the fans all day, 103 (19%) used fans only when necessary, and 358 (66%) did not have fans. In the WNVN group 111 (28%) stables had fans, 54 (13%) used the fans all day, 49 (12%) used fans only when necessary, and 290 (72%) did not have fans. Fans were significantly correlated with WNV in the logistic regression (Table 2-5). The presence of fans increased the risk of WNV by 80%. The duration of use (only when necessary or all the time) was not significant in the logistic regression.

Mosquito larval habitats are associated with standing water, thus the retrospective survey attempted assesed the types of water that were present on the property to determine their significance in relation to WNV status in horses (Table 2-6). No association was detected for the type of water source or the presence of temporary or permanent water bodies on the farm in the X^2 analysis. In the logistic regression model, natural water on the property has a protective effect of reducing WNV by half (Table 2-5). The type of water associated with risk could not be determined because respondents were allowed to mark multiple answers on the questionnaire.

Debris and tree canopy can also provide adult mosquito resting habitat; however, there was no association with the presence of debris piles or tree canopy in the pasture or near the barn.

The presence of dead birds on the property was significantly (P = 0.003) associated with WNV activity. These birds were reported by the owner and may or may not have been postitive with WNV. Additionally, other horses becoming ill with WNV on the property were also significantly (P = 0.048) associated with WNV-positive horses.

The respondents were asked to mark their perceived level of mosquito activity (none, minimal, moderate, and severe). The level of mosquito activity on the farm was significantly (P = 0.016) correlated with an increased risk of WNV in the logistic regression model. The minimum level of mosquito activity increased risk of WNV by 128% when compared to no activity (Table 2-5). The other levels of mosquito activity also showed an increased risk, as indicated from the odds ratio, but were not associated with an increased risk of WNV strongly enough to be significant in the regression model.

Discussion

The purpose of this study was to describe the extrinsic risk factors associated with WNV infection and to develop recommendations for prevention of WNV infection in Florida horses. This study is distinctive because it is an extensive analysis of risk factors performed on WNV positive and negative horses. West Nile virus was first reported in Florida during June of 2001 and the WNV vaccine was conditionally released for use in Florida and throughout North America in August of 2001. The implication of the timing of virus introduction in Florida and vaccine release is that the Florida equine population represented a completely naïve population relative to WNV exposure in 2001. In the northeastern US, horses had been exposed to WNV since 1999, and in the western US, the vaccine was released prior to the first reported horse cases of WNV. In the face of the WNV outbreak many Florida horses were vaccinated. The results of

this study suggest that horses vaccinated during the summer of 2001 may not have had adequate time for optimal immunity to develop following vaccination and prior to the onset of clinical symptoms. In fact in a naïve population, there may even be a negative impact of vaccination at the time of exposure.

Since the release of the WNV vaccine in 2001, there have been high numbers of horse cases in western regions of the United States that did not experience WNV activity until after the vaccine was available. Just as cases of eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) occur annually in Florida despite the availability of a vaccine, there is likely to remain a group of unvaccinated horses that are susceptible to WNV infection each year. Furthermore, the continued annual transmission of WNV in the U.S. despite the availability of three vaccines against WNV supports the argument that the virtual disappearance of WNV transmission to horses in Florida since 2004 is related to WNV transmission patterns in Florida rather than to a completely protected equine population.

The arboviral case information form (ACF) allowed tracking of disease and gathering of signalment (clinical signs and symptoms) and demographic data for the 2,824 horses tested from 2001 to 2005 in the state of Florida. The IgM capture ELISA was the most commonly used test to classify WNV disease status in horses. The gold standard for arbovirus diagnosis is regarded as neutralizing antibody testing (PRNT) (Farfan-Ale et al. 2006). Neutralizing antibody testing cannot be used reliably for horses that have been vaccinated because the horse vaccine consists of formalin-inactivated whole-virion virus eliciting an IgG and neutralizing antibody response (Porter et al. 2004).

A significantly higher number of geldings than mares or stallions were WNVD in this study. Epp et al. (2005) recorded the gender of horses affected by WNV in Saskatchewan, but

did not report a significant relationship between WN disease incidence and gender. Other studies have reported a higher incidence of WNV in male horses than females {Tber A.A., 1996 3 /id} Ostlund et al. 2001). In a serosurvey in France there were no significant differences based on gender in subclinical WN disease in horses (Durand et al. 2002). In a study of risk factors of death in clinically affected horses, males were more often diagnosed with WNV; however, females were 2.9 times more likely to die from WNV than males {Salazar, 2004 342 /id}. Testosterone levels in males may attract more mosquitoes and increase the likelihood of WNV transmission to males. Alternatively, stable conditions may vary by gender, for example stallions may be stabled indoors more often then geldings, thereby increasing mosquito exposure to animals pastured outdoors more frequently.

A thorough analysis of the vaccination history of horses tested in this study was made. The vaccine had a protective effect against WNV infection in those horses that were vaccinated two times in a period of two weeks to one year prior to infection. In contrast Salazar et al. (2004) reported that one vaccine dose of the same vaccine provided protection. It is possible that the horses in my analysis did not experience the same amount of time frame after the single vaccine and before WNV exposure, as did the horses in the Salazar et al. study. The results from both of these studies are only applicable to the formalin inactivated whole virion vaccine, which was the only available vaccine on the market for the duration of the owner survey portion of this study from 2001 to 2003. In 2008 there are three licensed vaccines on the market with different modes of action, which result in varying duration of immunity that may interact with other environmental factors differently from the outcome reported in the present study.

In addition to vaccination, arbovirus prevention measures include barrier protection and repellent use. Barrier methods, such as flysheets, were not often used and were not significantly

associated with WNV infection. Repellents were often used and included pyrethins, natural oils, and Skin-so-Soft® lotion. DEET is not and active ingredient in insect repellents sold for use on horses due to documented adverse skin reactions (Palmer 1969). The most common type of repellent applied to horses was a pyrethrum-based insecticide. There was not a significant association with repellent use and WNV infection; however the linear regression was close to significant for a protective effect against WNV infection, and the association may merit further study.

Fans in the stable greatly increased the risk of WNV infection. The important factor in the increased risk of WNV was only whether fans were used or not. It is likely that there is an association of duration of fan use and disease outcome. Duration of fan use may have been confounded by other variables because this factor was significant in multivariable analyses. Most fans used in stables are small, non-industrial indoor fans. The strength of these small fans may not be enough to create a breeze that will prevent mosquito flight and blood feeding on the horses in the stable. However, the small fans do aid in the dispersal of CO_2 and other chemical odors that act as cues for host seeking mosquitoes (Bowen, 1991). When located inside a stall, the fans may increase the range at which mosquitoes can detect the horse and attract them in from a longer distance to blood feed. Fan use likely corresponds with human activity in the barn. Because of fire risks, workers are likely to only use the fans in the day when they are present in the barn. Fan use may not correlate with highest mosquito activity periods (between sunset and sunrise) but probably serves to effectively disseminate strong odors from the barn into the surrounding environment. If the mosquitoes can detect host odor from a greater distance, a higher number of mosquitoes may be able to successfully locate and blood feed on the stabled horses.

Natural water on the property was significantly associated with a reduced risk of WNV infection. The most common type of water on the property was a stream, river, or moving body of water, which was not suitable for mosquito larval development. This resulted in the apparent protective association of natural water on the property. The presence of a stream on the property was associated with a reduced risk of WNV infection in horses, but no other types of water were significantly associated with WNV infection. No other association was detected for the type of water source or the presence of temporary or permanent water on the property. This was unexpected due to the close association of mosquito larval habitats and adult mosquito abundance with rates of arboviral transmission. Debris and tree canopy can also provide suitable adult mosquito resting habitats; however, there was no association with the presence of debris piles or tree canopy over the pasture or barn with WNV infection.

Abundant mosquito populations are a necessary prerequisite for transmission of WNV (Zyzak et al. 2002). The minimum perceived level of mosquito activity was significant for increased risk of WNV infection. Other levels of perceived mosquito activity had an odds ratio demonstrating an increased risk for WNV infection but were not significant in the linear regression model. Important factors associated with epidemics are mosquito population size and age (Lord and Day 2001), mosquito infection rates, and mosquito transmission rates (Reeves et al. 1961, Rutledge et al. 2003). When compared to no mosquito activity, a minimum level of mosquito activity was associated with a 128% increased risk of WNV infection (Table 2-5). The correlation of mosquito activity and WNV transmission is associated with the minimum infection rates (MIRs), blood feeding activity, and transmission rates, which are all key factors in viral transmission to vertebrate hosts.

Stalls constructed from solid wood or cement were associated with a higher risk of WNV infection in horses than were stalls constructed from boards with openings or open sheds. In a study performed by the USDA in 1999 and 2000, pasture management was associated with higher rates of WNV disease than indoor stabling. The previous study examined horses exposed in the northeastern U.S. Stalling in a hot, humid environment may actually provide an environment for at least equal feeding of mosquitoes compared to the pasture. A solid stable construction, combined with lack of environmental temperature control may be putting horses at an increased risk of mosquito exposure. Additionally, the solid stall construction may provide resting sites for adult mosquitoes.

Guptill et al. (2003) showed avian deaths were associated with an increased level of WN viral activity and could serve as a warning of human infection. In my study, dead birds on the property were a powerful indicator of WNV transmission risk (Table 2-5). If a dead bird (regardless of WNV disease status) was seen on the property there was a strong indication of WNV activity in the immediate area (assuming that the bird did not disperse far from its original infection site) and was associated with a 97% increased risk of WNV infection to horses. Other ill equids (due to WNV) on the property were similarly a good risk indicator (Table 2-5).

This study could have been improved by including a group of clinically normal horses as controls in the analysis. All horses in this study showed some type of clinical manifestation consistent with a neurological infection. Some of the horses in the WNVN group were positive for EEEV and this may have somewhat reduced the power of the study to evaluate risk of WNV.

In conclusion, WNV disease in Florida horses appears to be primarily related to vaccination status. As of 2008, there are three efficacious vaccines available and if horses receive two doses according to manufacturers instructions, the incidence of equine WNV could

be effectively controlled (Ng et al. 2003, Seino et al. 2007). The use of fans in the stable should be examined more closely with a focus on the type of fan, time of day used, and other factors that may provide a more conclusive correlation with use and WNV infection. Some other variables that warrant further study are the use of insecticide or repellent and the canopy cover which both had close to significant associations in the regression model with WNV infection. In areas of high vector activity, reduction of vector larval habitat and limiting horse exposure to mosquitoes remain important prevention methods for Florida equines. Dead birds on the property and other ill equids should be noted and considered an indicator of viral activity in the area. Precautions against mosquito exposure should be taken to protect horses on the property when dead birds have been reported nearby, especially if the horses are unvaccinated.

Table 2-1. Outline of information submitted by Arboviral Case Form (ACF) and by retrospective mail survey (RMS). Veterinarians submitted data on all horses tested for arboviruses in the state via the ACF.

Farm/Sample Submission	Source
County of origin	ACS
Horse origin	ACS
Sample(s) submitted	ACS
Date of onset of clinical signs	ACS
Date of testing	ACS
Signalment and History	
Age	ACS
Sex	ACS
Breed	ACS
Use	RMS
Arbovirus Prevention	
Vaccination	ACS/RMS
Frequency of vaccination	RMS
Fly spray frequency	RMS
Fly spray type	RMS
Barrier protection with flysheet	RMS
Stable Characteristics and Farm Ecology	
Type of stable structure	RMS
Duration of outside activity	RMS
Frequency of stall cleaning	RMS
Manure handling	RMS
Presence of debris	RMS
Tree canopy characteristics	RMS
Vector activity (mosquito abundance)	RMS
Presence of dead birds	RMS

These data were submitted to the Florida Department of Agriculture and Consumer Services as part of reporting requirements in the state of Florida for all horses exhibiting symptoms of encephalitis. Another source of data was the RMS filled out by the owner of the horses tested; the surveys were returned to the College of Veterinary Medicine.

Table 2-2. Total number of horses exhibiting signs of encephalitis and test results for WNV from 2001 to 2005

	ACF WNVD	ACF WNVN	RMS WNVD	RMS WNVN
2001	651	382	234	138
2002	643	323	265	95
2003	73	429	35	169
2004	7	173		
2005	12	131		
Total	1386	1438	534	402

(Source: ACF) and number of mail surveys (RMS) returned from owners of horses tested between 2001 and 2003.

Table 2-3. Age of WNVD horses in Florida 2001-2004

Ages	2001	2002	2003	2004	Total
<1	7	34	2	0	43
1-2	133	24	9	1	167
2-3	122	28	4	0	154
3-4	46	34	1	0	81
4-5	23	29	5	1	58
5-6	16	21	4	0	41
6-7	22	28	4	1	55
7-10	61	101	20	2	184
>10	145	9	20	2	176
Total	575	308	69	7	959

Source: ACF

Table 2-4. Gender of horses tested for WNV from 2001-2004.

	Female	Male	Stallion
2001			
WNVD	279	273	59
WNVN	156	134	49
2002			
WNVD	310	273	32
WNVN	22	22	20
2003			
WNVD	37	34	2
WNVN	222	144	95
2004			
WNVD	0	100	0
WNVN	110	88	36
2001-2004			
WNVD	626	680	93
WNVN	510	388	200

Source: ACF

Table 2-5. Results of logistic regression analysis factors associated with WNV among horses with clinical signs in the state of Florida between 2001 and 2003.

Variable	Category	OR	95%CI	P value
Vaccination				<0.001
	2001	1.68	1.12-1.83	
	2002	0.34	0.67-0.93	
	2003	0.42	0.31-0.00	
Vaccinated 2wks-6mo				0.002
	Yes	0.06	0.04-0.82	
	No	1		
Received two doses				<0.001
	Yes	0.48	0.01-0.69	
	No	1		
Protective vaccine				<0.001
	Yes	0.18	0.09-0.98	
	No	1		
Fans in stable				0.013
	Yes	1.79	1.22-2.53	
	No	1		
Natural water				0.001
	Yes	0.48	0.31-0.75	
	No	1		
Dead birds				0.003
	Yes	1.97	1.27-3.47	
	No	1		
Other animals ill				0.048
	Yes	2.42	1.01-5.79	
	No	1		
Mosquito activity				0.016
	None	1		
	Mild	2.28	1.35-3.52	
	Moderate	1.67	0.57-1.74	
	Severe	1.18	0.82-1.20	

Table 2-6. Stable characteristics and farm ecology for horses classified as West Nile virus diagnosed (WNVD) or negative (WNVN) by the Florida Department of Agriculture and Consumer Services 2001 to 2003.

Variable	WNVD	WNVN
Where was horse primarily turned out? (# responses [%])		
Pasture	359 (66)	250 (62)
Grass paddock	66 (12)	48 (12)
Sand paddock	64 (12)	46 (11)
Unanswered	54 (10)	58 (14)
How often are the stalls cleaned? (# responses [%])		
Monthly	12 (2)	14 (3)
Twice a month	2 (0)	7 (2)
Weekly	33 (6)	20 (5)
Daily	240 (44)	157 (39)
Not applicable/unanswered	256 (47)	204 (50)
Are there fans in the stable? (# responses [%])		*
Yes	184 (34)	111 (28)
No	358 (66)	290 (72)
Unanswered	1 (0)	1 (0)
How often are the fans run? (# responses [%])		*
All the time	70 (13)	54 (13)
Only when necessary	103 (19)	49 (12)
Never	0 (0)	1 (0)
Not applicable/unanswered	370 (68)	298 (74)
What is the stable made of? (# responses [%])		*
Boards with openings	158 (29)	131 (33)
Solid wood or cement	146 (27)	79 (20)
Open shed	73 (13)	60 (15)
Unanswered	166 (31)	132 (32)
After rain, is there temporary standing water on the property? (# responses [%])		
Yes	257 (47)	192 (48)
No	285 (52)	209 (52)
Unanswered	1 (0)	1 (0)
Tree canopy cover (# responses [%])		
None	65 (12)	52 (13)

Table 2-6. Continued

Over barn	38 (7)	29 (7)
Over pasture	227 (42)	160 (40)
Over both	163 (30)	110 (27)
Unanswered	50 (9)	51 (13)
How many debris piles exist near the stable, or area of horse activity? (# responses [%])		
0	291 (54)	189 (47)
1	128 (24)	82 (20)
2	34 (6)	26 (6)
3	11 (2)	11 (3)
More than 3	27 (5)	33 (8)
Unanswered	52 (9)	61 (15)
Severity of mosquito/fly activity (# responses [%])		*
None	72 (13)	40 (10)
Mild	208 (38)	148 (37)
Moderate	145 (27)	107 (27)
Severe	71 (13)	37 (9)
Unanswered	47 (9)	70 (17)
Were there any dead birds on the property? (# responses [%])		*
Yes	98 (18)	69 (17)
No	444 (82)	332 (83)
Unanswered	1 (0)	1 (0)

* Significant $P<0.05$

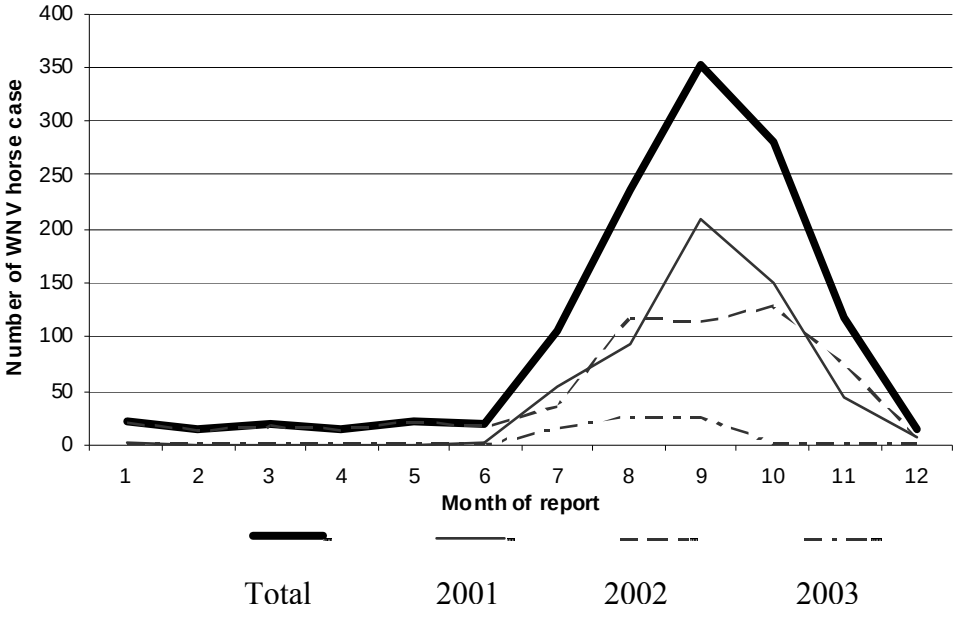


Figure 2-1. Total WNVD horse cases reported in Florida between 2001-2003.

CHAPTER 3
MOSQUITOES COLLECTED IN LIGHT TRAPS, RESTING BOXES, AND HORSE-BAITED TRAPS IN NORTH FLORIDA

Introduction

West Nile virus (WNV; family *Flaviviridae*: genus *Flavivirus*) is a pathogen that is primarily maintained between birds and mosquitoes in enzootic transmission cycles, but is also sometimes transmitted to mammals, including horses and humans (Petersen and Roehrig 2002). The virus and disease have been present in the United States since 1999 and have continued to spread throughout North and Central America and throughout the Caribbean Basin causing annual outbreaks (Reisen and Brault 2007). An efficacious horse vaccine has been available since 2001, but horse cases continue to occur annually throughout the transmission zone. In Florida alone, 1,082 horses were diagnosed with WN infection between 2001 and 2007 (USDA-APHIS 2007). Florida has a large equine industry with over 299,000 horses in the state potentially at risk for WNV infection (FDACS 2007). Estimates of asymptomatic (subclinical) WNV infection in horses have ranged from 1.2% (Lorono-Pino et al. 2003) to 58% (Durand et al. 2002). Of the clinically infected horses, 35-40% of the cases result in death. West Nile virus is a reportable disease in Florida but because a large proportion of infected horses do not show symptoms of infection it is likely that horse cases are underreported.

West Nile virus is maintained in enzootic foci where mosquito and bird populations are in close proximity (Campbell et al. 2002). The primary (enzootic) cycle involves avian hosts and ornithophilic mosquitoes and the secondary cycle involves non-avian hosts and epizootic vector mosquitoes which are sometimes linked between both cycles. Vertebrate hosts that facilitate WNV epidemics are termed amplification or reservoir hosts (Kilpatrick et al. 2006). Amplification hosts spike a high viremia for a short duration of time; reservoir hosts may sustain a low level viremia for a long duration and aid in maintaining the virus through periods of low

mosquito activity. Avian amplification hosts remain infective for WNV for one to three days (Komar et al. 2003). The transmission of virus between infectious amplification birds and vector mosquitoes (primarily *Culex* spp) results in amplification of the virus. After sufficient amplification in the bird population, the virus escapes its focus when infective mosquitoes disperse into habitats where they may come into contact with a susceptible non-avian host. Epizootic vectors such as *Culex nigripalpus* (Theobald), *Culex salinarius* Coquillett, *Aedes vexans* (Meigen) and *Coquillettidia perturbans* (Walker), which are opportunistic feeders, transmit WNV to horses and humans (Campbell et al. 2002, Samui et al. 2003). Isolations of WNV from natural mosquito populations in Florida have been reported in *Cx. nigripalpus, Mansonia titillans* (Walker), *Ochlerotatus taeniorhynchus* (Wiedemann), and *Deinocerites cancer* Theobald (FDOH 2007, CDC 2007). *Culex nigripalpus* is considered an epidemic and epizootic vector of St. Louis encephalitis virus (SLEV; family *Flaviviridae*, genus *Flavivirus*) in Florida, which shares a similar epidemiology to WNV (Shaman et al. 2005, Zyzak et al. 2002).

A thorough knowledge of the biology, ecology, and behavior of mosquito vectors is essential for understanding WNV transmission, amplification, epizootics, and epidemics. The blood feeding behavior of many mosquito species has been studied (Apperson et al. 2002, Lee et al. 2002, Ngo and Kramer 2003) and this information combined with mosquito susceptibility to viral infection and mosquito trapping data can help identify possible mosquito vectors of WNV to horses.

Livestock-baited traps have been widely used to identify the presence, seasonal abundance, and host preference of mosquitoes. One of the first portable stable traps used for the collection of mosquitoes was the Magoon trap, designed in 1935, and with various modifications, the trap remains widely used today (Service, 1976). Samui et al. (2003) classifies a mosquito species as

an important horse feeder if it frequently enters a horse-baited stable trap and if a large percentage of the individuals entering the horse-baited stable trap are blood fed when they are collected. In areas of eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) transmission, a mosquito that is a competent EEEV vector, feeds on a horse may serve as an epizootic vector from the amplification host to the horse (Samui et al. 2003). Data from horse-baited traps can help identify which mosquito species are present in a locality and which mosquito species feed on horses. An understanding of these mosquito/host interactions can help augment existing knowledge of potential vector species for arboviruses to infect horses. Of particular interest in this are those species that are physiologically competent for EEEV or WNV, have had field isolations of EEEV or WNV, and have been temporally associated with EEEV or WNV transmission foci.

The purpose of this study was to determine the identity and seasonal abundance of mosquito species attracted to horses in a study area in north Florida. To accomplish this, a two-year mosquito surveillance project was designed to provide information about the seasonal abundance and spatial distribution of mosquito species near horses maintained at the study area. These data were collected to evaluate mosquito seasonal variability and host seeking behavior compared to WNV transmission (n = 1) and EEEV transmission (n = 10) to horses in north Florida in 2005 and 2006. Three clinical WNV horse cases were reported at the study site in 2001 and, based in part on this observation, the site was chosen for the present study. The relative abundance and species composition of mosquito fauna collected from and around horses at the north Florida study site are reported here and compared with light trap and resting box collections made at the same site during the same time period.

Materials and Methods

Study Site and Mosquito Collection Protocol

The study site for this project was located at the University of Florida Veterinary School, in Alachua County in north central Florida (29°38' N, 82°20' W). Four CDC light traps (John W. Hock Company, Gainesville, FL) (Sudia and Chamberlain 1988), four resting boxes (Moussa 1966), and a horse-baited stable trap (Bates 1944) were used to collect mosquitoes. The site was chosen based on a history of WNV transmission to horses (three clinical cases in 2001), abundant mosquito habitats, a reliable population of horses (n = 50) permanently pastured at the site, and a ranch-style operation surrounded by urban development.

The study area was mostly open grass bordered by a sylvan habitat supporting nighttime mosquito flight activity and providing many mosquito-resting habitats. Bivens Arm Nature Park bordered the site to the south. The nature park is a 57-acre urban wetland (43 acres of aquatic habitat) bordered by upland mixed forest (14 acres) (Fig. 3-1). The main feature of the park is a lake surrounded by a live oak hammock. A 1500 sq-ft retention pond was located on the north side of the study site. After a rainfall event, standing water would accumulate in the pastureland at the site. The water would drain slowly into the retention pond over a period of several days unless rainfall continued.

The research horses (n = 50) at the site were kept in outdoor pastures and the property had an 80-stall equine hospital on the west side. The hospital's outside lights were left on at night and veterinary students and doctors had access to the hospital and were sometimes present throughout the night. Both of these factors may have influenced the abundance and species composition of mosquitoes around the hospital at night.

In 2005, the stable trap was located in a single barn containing six stalls with a large hallway in the middle. Each stall had two 30.5cm by 30.5cm windows, one opening to the

outside of the stable and the other opening into the hallway. One stall (36.5m^3) was modified into a horse-baited stable trap following the design of Bates (1944) (Figure 3-2). Mosquito netting was secured to all sides of the stall and across the ceiling. Two horizontal 30.5cm baffles were placed along the windows on the outside and inside of the stall 1.21 m from the ground (Figure 3-2). The baffles consisted of a cut foam mattress pad glued to the side of the stall to make a V-shaped opening of 20-cm to the outside and converging to a 2.5-cm opening into the trap. The floor of the stall was covered with sawdust that was cleaned after each use.

The barn used in 2005 was not available for the study in 2006, so a change in stall location occurred. The design of the stall was almost the same, only small modifications were made and the stall was located 14 m south of the stable used in 2005 (Figure 3-1). The stall was a standard portable stall design measuring 3.65m^3 x 3.65m x of 3.65m on the low side angled up to 4.26m on the high angle side. The portable stable trap was large enough to house an adult horse. The bottom half of the trap was constructed of wooden boards and the upper half of vertical steel bars with a slanted tin roof. To modify the stall following the design of Bates (1944), the entire stall was sealed with mosquito netting. To construct the entrances into the trap, 30cm lengths of PVC pipe with a 15.2cm outer diameter were cut in half longitudinally and secured along two sides of the stall at a height of 1.21 m (Figure 3-3). The diameter of the openings were 15.2 cm to the outside with a 1.9 cm opening cut along the inside of the pipe facing into the stall to act as a baffle allowing mosquitoes entrance into the stall and limiting escape. The floor of the stable trap was covered with sawdust and was cleaned after each use.

The study commenced on October 6, 2004 and ended on December 5, 2006. The stable trap collections were conducted from May 2005 through November 2005 for the first stable trap and from May 2006 through November 2006 for the second. Because the horse had freedom to

move about in the stall and was provided with food and water, the stable trap could be run overnight (IACUC approval #E248). Four CDC light traps (John W. Hock, Gainesville, Fl) baited with approximately 1 kg of dry ice were placed in different habitats at the site. In relation to the stable trap, the light traps were placed as follow: 1) in a highly wooded area (300 m south), 2) by a 1,500 sq-ft retention pond (50 m north), 3) in an oak stand (50 m west) and 4) by the horse stable trap (10 m east). A resting box was paired with each of the four light traps (Figure 3-1). Each resting box was constructed of plywood in the form of a cube 30.5 cm on each side and one open side. The open side was covered with a square of mosquito netting that could be secured to the sides by Velcro. The cover was left open over night and in the morning secured to trap mosquitoes inside prior to aspiration. The outside of each box was painted black with acrylic paint, and the inside was a deep red color to provide a dark space for resting mosquitoes. All traps were set twice a week at about 1600 hr. Traps were retrieved the following morning at approximately 0800 hr.

The stable trap and the resting boxes were aspirated with a backpack aspirator (Dvacc, John Hock, Gainesville, FL) followed by a small hand held aspirator (Bioquip, Rancho Dominguez, CA) to reach into the smaller spaces. Collection bags from each trap were placed individually in a -70°C freezer and then the contents were transferred to Petri dishes, labeled, and stored at – 70°C until the mosquitoes were identified to species on a chill table and sorted into pools by species, date, and trap type. All blood fed mosquitoes were stored separately, and empty females were stored in pools of up to 50 and tested for virus (results of the viral analysis are reported in Chapter 4).

Fluorescent Mosquito Release and Recapture

To evaluate mosquito escape from and entrance into the two stable traps used in this study eight marked mosquito release trials were performed. In 2005 and 2006, colony reared *Culex*

quinquefasciatus females (USDA-Gaineseville, Fl) were colored red and green with Dayglo fluorescent dye. The mosquitoes were knocked down by cooling to 4°C and gently shaken in a container lightly coated with fluorescent powder to dust the mosquitoes and cover them in the dye. During these studies in 2005 (n = 4) and 2006 (n = 4), 100 green mosquitoes were released outside of the stall and 100 red mosquitoes were released inside the stall at 1700 hr. In 2005, red mosquitoes were released inside the stall once in the presence of a horse and three times in the absence of a horse. In 2006, mosquito releases were made three times in the presence of a horse and once in the absence of a horse. In 2005, an un-baited light trap was set in an adjacent stall next to the stable trap. No other horses were present in the stalls next to the stable trap during these tests.

Colonized *Culex quinquefasciatus* were chosen for the mosquito release trials because this species naturally occurred at the study site and a laboratory colony was easily assessable. *Culex quinquefasciatus* is an opportunistic feeder (Elizondo-Quiroga et al. 2006). Females from the laboratory colony were fed bovine blood, demonstrating their willingness to feed on mammalian blood and colony males and females were provided continual access to a 10% sugar solution for flight and maintenance energy. Prior to experimental release, the mosquitoes were provided only water for 24h to ensure maximum host seeking behavior upon release.

Results

During the study 45,851 mosquitoes were captured in the three trap types: light traps, 45,271; horse-baited stable traps, 526; and resting boxes, 55. Twenty-three mosquito species were captured in light traps, seven in the horse-baited stable traps, and three in the resting boxes. Totals for the nine most abundant species are illustrated in Figure 3-4. All seven species collected inside the horse-baited trap blood fed on the horse. These were all confirmed as horse blood meals by PCR analysis (Table 3-1).

Fluorescently dyed *Culex quinquefasciatus* females were released four times each year to determine the recapture rate inside the stable trap and to validate mosquito entry and exit from the stable trap. The number of mosquitoes recaptured inside the stall when a horse was present, varied from 16% (16/100) to 41% (41/100). When no horse was present the number recaptured the following morning varied from 1% (1/100) to 5% (5/100) (Table 3-2). For mosquitoes released outside the stall, there was an entry and recapture rate of varying from 3% (3/100) to 7% (7/100) when a horse was present. None of the marked mosquitoes entered the stall when a horse was not present nor were any of the marked mosquitoes recaptured in the un-baited light trap located in the adjacent stall.

During the first 13 months of the study (Oct. 2004 to Oct. 2005) *Cx. nigripalpus* was the most abundant mosquito species collected. The high *Cx. nigripalpus* numbers were recorded during the months of October and November 2004 (Figure 3-5). The next most abundant mosquito species collected during the first half of the study were *Anopheles crucians* (Wiedemann), followed by *Ma. titillans, Oc. infirmatus, Cx. erraticus,* and *Cx. salinarius* (Figure 3-4). After November 2004, the number of mosquitoes captured decreased dramatically. In March of 2005 *An. crucians* populations began to increase followed by an increase in April 2005 of *Oc. infirmatus*. The abundance of both of these species decreased after July 2005. The number of *Ma. titillans* increased in the early fall and remained high until the end of December 2005 (Figure 3-5). When mosquito numbers and species composition were compared between the light trap and the horse- baited trap (mosquitoes collected inside the horse stall), a preference of *Ma. titillans* for the horse was observed over the light trap (Table 3-3).

Mosquito abundance and species diversity patterns observed at the study site during the second 13 months of the study (November 2005 to November 2006) (Figure 3-5). The most

abundant mosquito species collected during this time period was *Ma. titillans,* followed by *An. crucians*, and *Cx. erraticus.* During 2006, females of three mosquito species entered the horse-baited stable trap: *Ma. titillans*, *Cq. perturbans*, and *Cx. erraticus*. Of the three species that entered the trap, only *Cq. perturbans* was collected in higher numbers in the horse-baited stall than in the light trap located near it (Table 3-3).

A comparison of the total number and species composition of the mosquito collections made during 2005 and 2006 appears in Table 3-4. *Culex nigripalpus* numbers in all trap collections declined dramatically from 10,530 in 2005 to 135 in 2006. *Culex salinarius* also declined from 1,494 in 2005 to 498 in 2006. *Culex erraticus* was much more abundant the second year and increased from 397 in 2005 to 3,108 in 2006. *Culex erraticus* was collected throughout the entire summer of 2006. *Mansonia titillans* was abundant during the autumn of both years. *Mansonia titillans* was less abundant in 2005 (n =2,153) than in 2006 (n = 10,134). *Anopheles crucians* was abundant during the winter and spring of 2005 (n = 2,979) and 2006 (n = 4,633). Its numbers declined in early summer and it virtually disappeared by August of both years.

Discussion

Seven of the 23 mosquito species collected during this study entered the horse-baited stable trap. The four light traps used during this study were responsible for 98% of the total mosquito catch. The light trap located next to the stable trap collected approximately the same number of mosquitoes as the stable trap did (light trap, n = 515; stable trap n = 526). Olson et al. (1968) found nine of the16 species that they studied entered a livestock-baited trap in a small farming community in Utah. In their study, light trap collections accounted for over 90% of the total trap catch with the exception of *Anopheles freeborni.* In contrast, Carpenter and Peyton (1952) found a total of 3,391 mosquitoes collected in light traps over a one-year period compared

with 65,323 mosquitoes collected in a horse-baited stable trap at the same site. Perhaps the discrepancies observed between this study and the other two studies discussed above are due to trap design and/or trap location. Because the total trap collections were about equal in the stable trap and the light trap, lends credence to a well-placed stable trap in this study.

All seven mosquito species collected in the horse-baited stable trap in my study have had associated field isolations of WNV (CDC 2007). This does not mean that all seven are 1) competent vectors and/or 2) important epizootic vectors, but field isolation of an arbovirus is one criterion used to identify a mosquito species that has had contact with a WNV positive host and is a potential arboviral vector. Polymerase Chain Reaction analysis of blood meals from the mosquitoes captured in the horse-baited stable traps used in my study confirmed that all of the mosquitoes that entered the stable trap blood fed on the horse contained in the trap (Table 3-1). All seven mosquito species (*Cx. salinarius, Cx. quinquefasciatus, Cx. erraticus, An. crucians, An. quadrimaculatus, Ma. titillans,* and *Cq. perturbans*) that entered the trap blood fed on the horse and can therefore be considered horse feeders at this study site (Samui et al. 2003). A goal of this study was to identify mosquito species that were likely to blood feed on horses at the site; further studies should be conducted to evaluate the potential of these mosquito species to transmit WNV and EEEV to horses in nature.

Mansonia titillans and *Cq. perturbans* showed a preference for the horse-baited stable trap compared with the adjacent light trap (Table 3-3). Olson et al. (1968) found the most abundant mosquito species present at their study site were the ones that entered the livestock-baited trap. In my study in 2006 *Cq. perturbans* composed only 6% of the total collection but was collected in about equal numbers in the stable trap (n =47) and the adjacent light trap (n = 43). *Mansonia titillans* was the most common mosquito captured in the stable trap during both years of my

study. The next most abundant species collected in the stable trap were *Cx. quinquefasciatus* in 2005 and *Cq. perturbans* in 2006. *Mansonia titillans* has been positive for WNV isolations in the field (CDC 2007); however, the detection of WNV in a given mosquito species does not mean that the species is a vector of WNV. Population density, host preference, feeding behavior, longevity, seasonal activity, viral susceptibility, and vector competence must also be considered when attempting to determine the status of a mosquito species as an important vector (Sardelis et al. 2001). *Mansonia titillans* will blood feed on avian hosts, but has a strong preference for mammals (Edman 1971). Members of the genus *Mansonia* have a long flight range of up to 2.5 km (Macdonald et al. 1990). A long flight range enhances the vector capacity of a bridge vector, if the species is an otherwise efficient and competent arboviral vector, by bringing the virus out of its amplification focus into different habitats where transmission can occur (Moncayo and Edman 1999).

Coquillettidia perturbans was collected frequently at the study site (n = 2,747). This species is considered an important bridge vector for EEEV (Chamberlain et al. 1954, Boromisa et al. 1987, Vaidyanathan et al. 1997). Eastern equine encephalitis virus has been isolated from pools of field collected *Cq. perturbans* (Nasci et al. 1993, Andreadis et al. 1998). This species is considered mammophilic, but also feeds on birds (Edman 1971). Because of blood feeding preference, collection in the horse-baited stable trap, and virus isolations field-collected females, *Cq. perturbans* may play a role in EEEV transmission in north central Florida. *Coquillettidia perturbans* has been demonstrated to be an inefficient laboratory vector of WNV and could occasionally play a secondary role in WNV transmission in the field (Sardelis et al. 2001). The *Culex* species that entered the horse-baited stable trap, did so in much lower numbers than their overall abundance as indicated by light trap collections at my study site. All of the *Culex* species

that entered the trap (n = 29 in 2005; n = 6 in 2006) blood fed on the horse indicating that these mosquitoes feed on horses in nature.

When a horse was present in the stable trap, 16% (16/100) to 41% (41/100) of fluorescently marked released mosquitoes were recovered the following day. Failure to recover 100% of the marked mosquitoes may have resulted from mosquito escape, or from death due to horse defensive measures including tail swipes, biting, and pawing with the feet. Very few (1%, 1/100 to 7%, 7/100) marked mosquitoes were recovered when no horse was present in the stable trap. It is likely the released mosquitoes actively and successfully searched for an exit from the unoccupied stable trap. Additionally, the fact that no mosquitoes (0/400) entered the stable trap when a horse was not present demonstrates that the horse itself acted as an attractant and that mosquitoes were not attracted to lingering odors left in the stall.

Very few marked mosquitoes entered the stable trap (16/400) when a horse was present during the mark-release-recapture trials. The collections inside the stall during both trapping seasons were about equal to the adjacent light trap for the same time period (Table 3-3). In mark-release-recapture trials, mosquito numbers recaptured are often low (Conway et al. 1974, Kramer et al. 1995, Reisen et al. 2003). The two reasons that affect recapture rates the most are wind speed and mosquito source (laboratory reared versus locally collected) (Reisen et al. 2003). The average wind speed on the nights of the marked mosquito release trials were between 4.52 mph and 15.07 mph.

Caution must be used when interpreting results from collections made by light traps, horse-baited stable traps, and resting box because the collections may not reflect true mosquito abundance (Huffaker et al. 1943). The most accurate mosquito population estimate is made by combining the results of multiple trap collection types (Huffaker et al. 1943, Bidlingmayer 1967)

to account for variation due to species biases of traps, biases due to trap location or the influence of meteorological conditions. Despite overall low mosquito numbers in the stable trap collections, the collection information can be useful when combined with other trap types used during this study. Simultaneously using various trapping methods can provide a reliable measurement of mosquito abundance and diversity (Huffaker, 1943, Bidlingmayer 1967).

Seven mosquito species fed on the horse maintained in a stable trap and the largest mosquito collections were made during the fall of both study years. *Mansonia titillans* was the most frequently collected mosquito in the horse-baited stable trap. *Mansonia titillans* and *Cq. perturbans* were the only two species at the site that were collected in higher number in the stable trap than in the adjacent light trap. Both of these species should be considered in future studies as potential vectors of WNV and EEEV, respectively, in north Florida. To my knowledge, laboratory studies have not been completed to evaluate the vector competency of *Ma. titillans* for WNV. Such studies would provide valuable information to supplement the results of my study. The possible role of *Ma. titillans* as a potential vector of WNV to horses should be investigated. *Coquillettidia perturbans* may play an important role in the transmission of EEEV to horses (Morris 1988) and may play a secondary role in WNV transmission (Sardelis et al. 2001). *Coquillettidia perturbans* is considered a bridge vector of EEEV (Crans and Schulze 1986, (Morris 1988) and it is likely that this species may play a role in EEEV transmission as an epizootic vector to horses in north central Florida.

The differences in mosquito numbers collected at the site each year are most likely due to variation in the weather conditions. In 2005 the weather was wetter than average (reported in chapter 4), and in 2006 Florida experienced a prolonged drought. The number of *Cx. nigripalpus* declined dramatically after the first few months of the study. The collections began in October

2004 and the hurricane season had been particularly active for Florida earlier in the year. Only a few weeks after the final hurricane of the season did trapping begin and it is possible that the large numbers of *Cx. nigripalpus* were correlated with the rainfall associated with this time period.

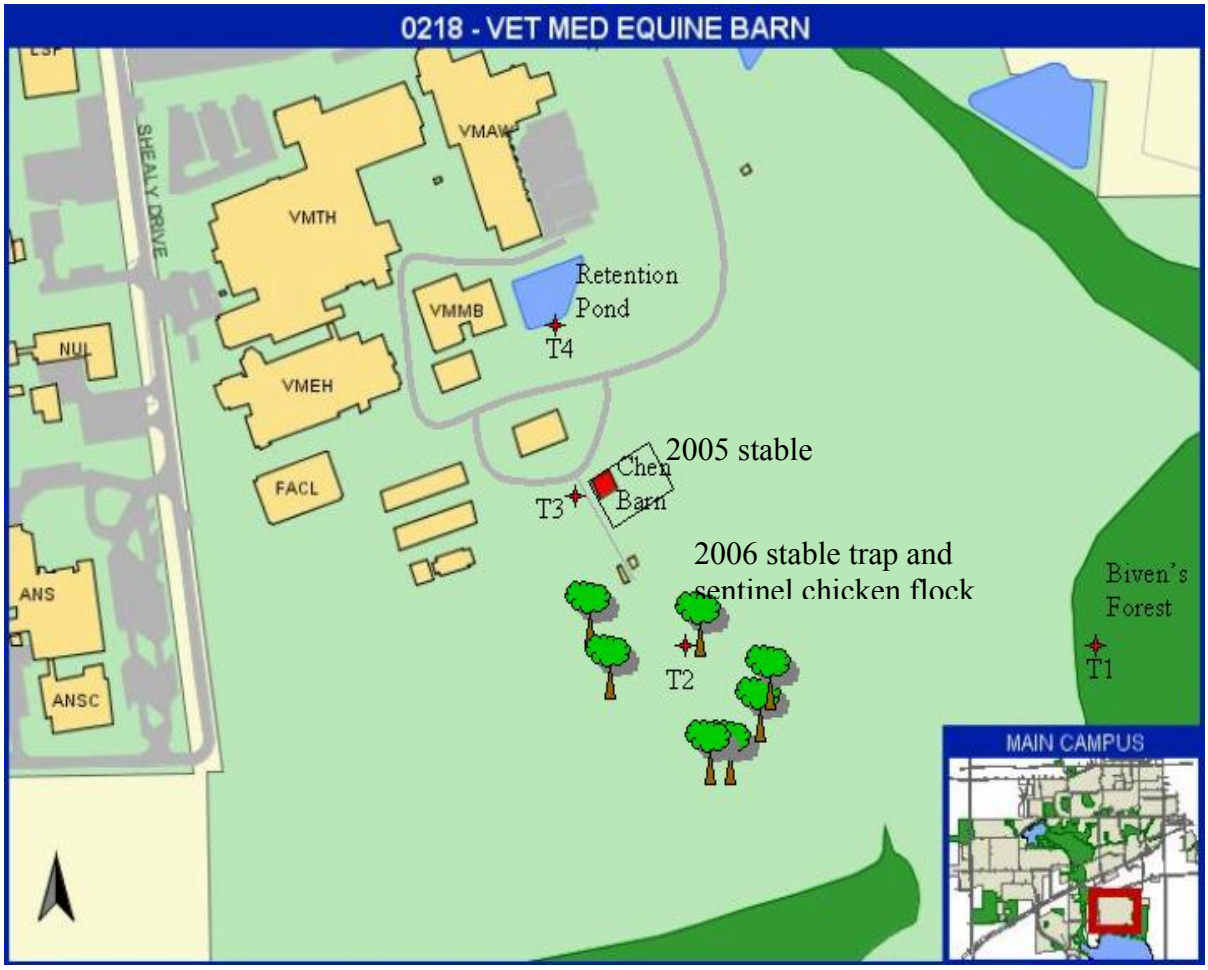


Figure 3-1. Location of the four paired light traps and resting boxes marked as T1, T2, T3, and T4. The Chen barn was modified for the stable trap in 2005, and a portable stall was erected 14m south of the Chen barn during the 2006 season. The sentinel chicken flock was located adjacent to the stable trap in 2006.

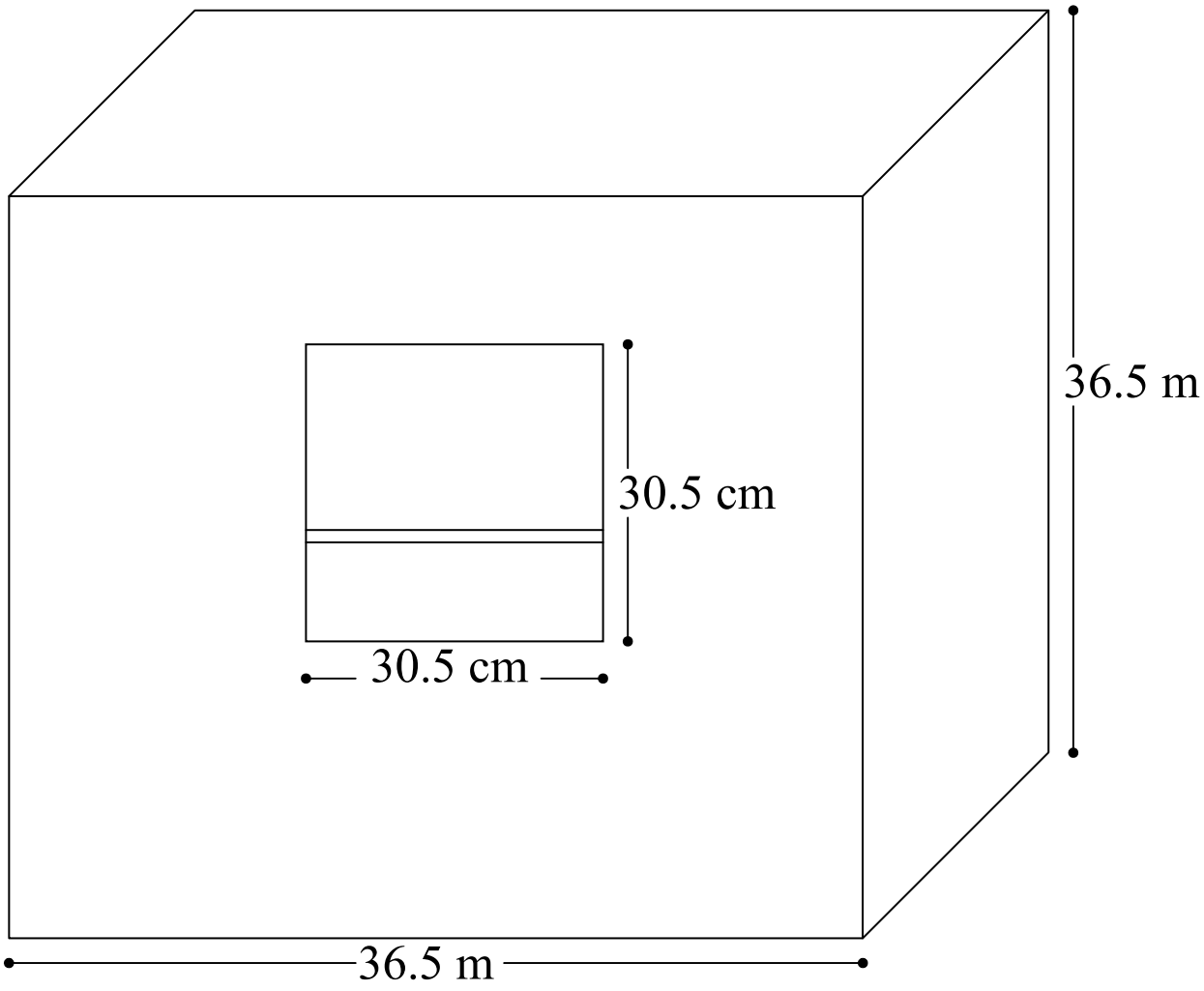


Figure 3-2. Measurements of the stall that held the horse in 2005. A single stall was modified for the trap in a six-stall barn.

Figure 3-3. Stable trap design 2006. 30 cm lengths of PVC pipe with a 15.2cm diameter cut in half with openings of 1.9cm were placed along two sides of the stall for mosquito entry. The rest of the trap was sealed with mosquito netting.

Table 3-1. Blood meals of mosquitoes collected in the horse-baited stable trap. A subsample (*n* = 50) of the total stable trap catch (*n* = 525) in 2005 and 2006 was analyzed. (Results of the blood meal analysis from mosquitoes collected outside the stable trap are presented in Chapter 5.)

Species	# tested	# confirmed (%)	result
Cx. salinarius	2	1 (50)	horse
Cx. quinquefasciatus	3	2 (66)	horse
Cx. erraticus	7	5 (71)	horse
An. crucians	1	1 (100)	horse
An. quadrimaculatus	4	1 (25)	horse
Ma. titillans	20	11 (55)	horse
Cq. perturbans	13	7 (54)	horse

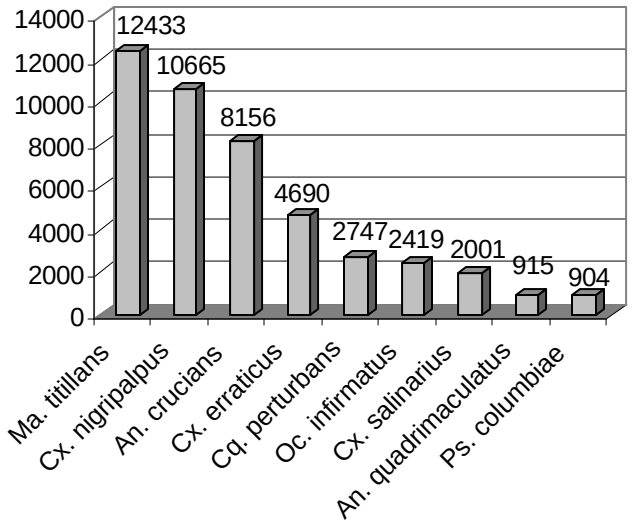


Figure 3-4. The species that represented at least 1% or more of the total trap catch between October 2004 and November 2006.

Table 3-2. Mosquitoes collected in the mark-release-recapture study in the horse-baited stable trap. Red marked mosquitoes were released inside the stall in groups of 100 each date. Green marked mosquitoes were released 5 m outside the stall in groups of 100 each date.

Collection Date	Horse present	Mosquito color	# recaptured inside stall	# recaptured outside stall
6/11/2005	Yes	Red	26	1
		Green	7	
9/16/2005	No	Red	3	
		Green	0	
9/23/2005	No	Red	1	
		Green	0	
9/30/2005	No	Red	4	
		Green	0	
9/16/2006	Yes	Red	41	
		Green	3	
9/21/2006	Yes	Red	16	
		Green	5	
9/28/2006	Yes	Red	25	1
		Green	1	
9/30/2006	No	Red	5	
		Green	0	

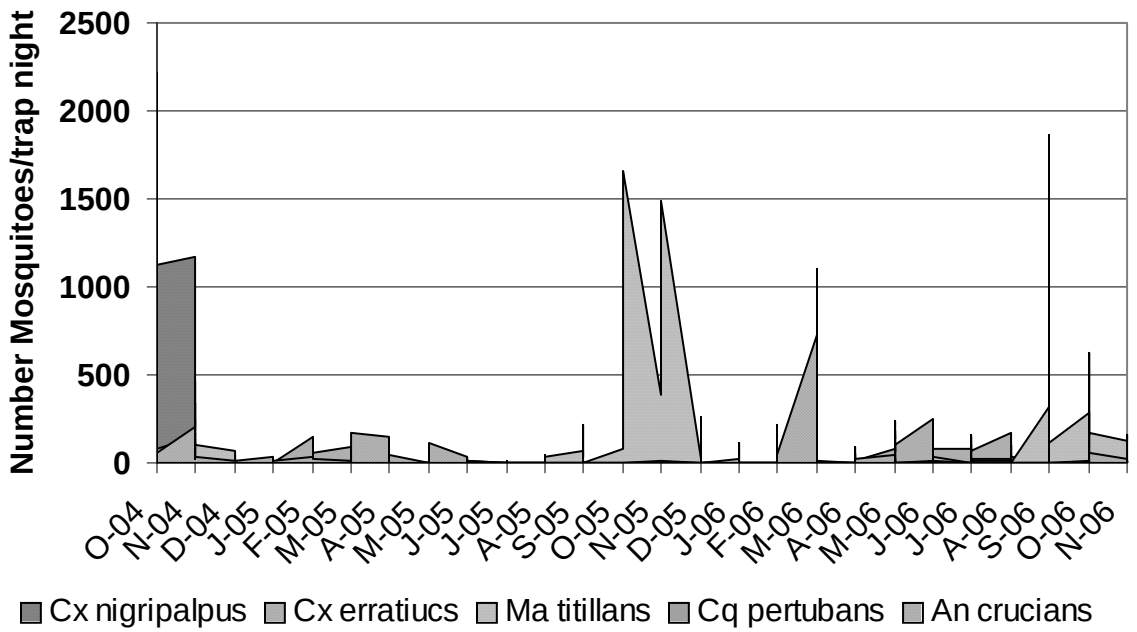


Figure 3-5. The most abundant mosquito species collected at the study site are represented over the two trapping seasons from October 2004 to November 2006.

Table 3-3. Comparison of mosquito catch in the horse-baited stable trap and an adjacent light trap. *Culex sp.* include *Cx. quinquefasciatus* (16) *Cx. salinarius* (3) and *Cx. erraticus* (10). *Anopheles sp.* include *An. quadrimaculatus* (9) and *An. crucians* (7).

May to Oct 2005	Horse Stall	Stable Light Trap	**May to Nov 2006**	Horse Stall	Stable Light Trap
Culex sp.	29	43	*Culex erraticus*	6	213
Anopheles sp.	18	22	*Mansonia titillans*	96	171
Ps. ciliata	1	0	*Cq. perturbans*	47	43
Mansonia titillans	330	13			
Cq. perturbans	1	9			

Table 3-4. Total number of five mosquito species caught each study year.

Mosquito species	2005	2006	Total
Ma. titillans	4,430	7,529	11,959
Cx. nigripalpus	10,530	135	10,665
Cx. erraticus	1,572	3,102	4,674
Cx. salinarius	1,498	500	1,998
An. crucians	2,991	5,158	8,149
Total	21,021	16,424	37,445

CHAPTER 4
ARBOVIRUS SURVEILLENCE: MOSQUITO POOLS, SENTINEL CHICKENS, AND HORSES.

Florida has had an arthropod borne virus (arbovirus) surveillance program in place since 1977 to track the amplification and transmission of mosquito-borne viruses including eastern equine encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) Highlands J (family *Togaviridae*, genus *Alphavirus*, HJ) and St Louis encephalitis virus (SLEV; *Flaviviridae*, genus *Flavivirus*) (Day and Stark 1996). The Florida Department of Health (FDOH), Division of Environmental Health, coordinates the surveillance program. The Interagency Arbovirus Surveillance Network reports to the FDOH and is composed of several local, state and federal agencies, which are involved with the surveillance and control of arboviral diseases.

Upon its arrival in the United States, West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) was easily added to the existing surveillance program with the addition of WNV-specific laboratory diagnostics. Because SLEV and WNV are antigenically related, cross-reactions are observed with some serologic tests and so plaque reduction neutralization testing (PRNT) is done to distinguish the two viruses. During Florida's first reported WNV transmission season (2001), virus was recorded in 65 of 67 counties (Blackmore et al. 2003). In both the northeastern U.S. and in Florida, wild bird mortality was the most sensitive viral activity indicator (Blackmore et al. 2003). In 2001, wild bird mortality was the first indication of viral presence in 54 of the 65 counties in Florida where WNV was detected (Blackmore et al. 2003). Due to the correlation of WNV-positive dead bird reporting and local WNV transmission, dead bird reporting has become a valuable surveillance tool in the United States (Eidson et al. 2001a, (Nasci et al. 2002).

Like SLEV, the natural cycle of WNV involves *Culex* mosquitoes and wild birds. However, unlike SLEV, WNV causes high rates of mortality in certain families of birds.

Members of the family Corvidae (crows, magpies, ravens, and jays) are particularly susceptible to fatal infection (Nasci et al. 2002). Chickens in the northeastern United States are not considered reliable indicators of human disease because seroconversions occurred after human cases had already appeared (Crans and Schulze, 1986 Cherry et al. 2001). For this reason, New York does not have a sentinel chicken program in place. In California and Florida, however, sentinel chickens are an indispensable component of arboviral surveillance because viral positives in chickens are closely associated with and predictive of human cases (Day and Lewis 1991, Reisen et al. 1994).

Horses are not currently bled as part of an active WNV surveillance program in the United States. In New York State horse positives were unreliable in the prediction of human cases of WNV (Trock et al. 2001). It is not yet known whether WNV surveillance in horses can predict human cases in Florida, but horse cases that are reported to local health departments are used as part of arbovirus surveillance. Blackmore et al. (2003) reported that the epicenter of the 2001 WNV outbreak in Florida horses was in Jefferson County. From Jefferson County, the outbreak spread east, west, and south to a total of 40 Florida counties with confirmed horse cases. In the counties reporting both horse and human cases, the horse cases preceded the human cases by one to four weeks (Blackmore et al. 2003).

Weather conditions greatly affect mosquito populations and consequently arboviral activity (Wegbreit et al. 2000, DeGaetano 2005, Pecoraro et al. 2007). Drought in the spring followed by summer rain is closely associated with transmission of SLEV and WNV in Florida (Day and Stark 1996, Day 2001, Shaman et al. 2005). Drought brings mosquito and bird populations into close proximity by limiting the available water. Epizootic amplification may occur under these circumstances if an abundant mosquito population is available to feed on susceptible wild birds.

Under conditions of prolonged drought, however, virus transmission is greatly reduced (Day and Lewis 1991). Several critical factors have been outlined as criteria that create a high epidemic risk for arbovirus transmission in south Florida. They include a large population of susceptible wild birds, severe drought in the spring followed by a wet summer, and the continuation of dry and wet patterns throughout the summer that focus virus transmission between the mosquito vectors and vertebrate hosts (Day and Lewis 1991). The predictive factors outlined here are based on aspects affecting *Culex nigripalpus* populations and dynamics. The weather pattern of rain followed by drought synchronizes the oviposition and blood feeding of *Cx. nigripalpus* and subsequently virus transmission (Day and Curtis 1993). *Culex nigripalpus* plays a major role in arbovirus transmission in the southern part of the state. It is necessary to make similar evaluations of the relationship between weather patterns and mosquito populations with virus transmission patterns in the northern part of the state where *Cx. nigripalpus* populations are typically much lower (Zyzak et al. 2002). Because *Cx. nigripalpus* is not as common in north Florida as it is in south Florida, weather conditions such as a mild spring may increase the population of *Cx. nigripalpus* in north Florida (Zyzak et al. 2002); or it is possible that other *Culex* species are playing a larger role in virus transmission.

There were three aims of this study. The first aim was to compare arboviral activity in Alachua County with the seasonal dynamics and abundance of mosquito species present at a north Florida site. The second aim of the study was to correlate the temporal patterns of viral activity and mosquito abundance with abiotic environmental factors including rainfall, temperature, and wind speed. The third aim was to examine mosquito abundance at four microenvironments within a site. To accomplish this goal a two-year surveillance project was designed to provide information about the seasonal patterns of arbovirus activity in relation to

the abundance of mosquito species at a study site in north central Florida. The data were collected to compare WNV, SLEV, and EEEV activity in north Florida with the mosquitoes collected at the study site. Arbovirus activity included sentinel chicken seroconversion and mosquito pool positives during the study period.

Materials and Methods

Sentinel Animals

The study site was located at the University of Florida Veterinary School, in Alachua County in north central Florida (29°38' N, 82°20' W) (see chapter 3 Materials and Methods). Three equines were used as arboviral sentinels at the site between May and November of 2005 and 2006. The sentinel horses had blood samples taken weekly to screen for the presence of antibody for WNV, EEEV, and HJ (IACUC approval # E312). Ten ml of blood was taken from the jugular vein with a vacutainer needle and drawn directly into a 10 mL blue top (3.8% Na citrate) vacutainer tube then centrifuged for 10 min at 600 g. The serum was tested at the University of Florida Emerging Disease Arboviral Research and Testing (EDART) laboratory with the Immunoglobulin M antibody-capture Enzyme-Linked Immunosorbent Assay (MAC-ELISA) for detection of viral antibody.

Two of the sentinel horses were blood donors (research horses kept on the veterinary school property for blood donation as needed, IACUC#A712) and were maintained permanently in an open field near a sylvan habitat (Figure 4-1). The third animal was a pony that was used twice a week as a bait animal in a stable trap. The pony was maintained in a pasture during periods when it was not housed in the stable trap. The pony was located on the opposite side of the study site, 600 m west of the blood donors (Figure 4-1).

Sentinel chickens were added to the protocol (IACUC approval # E248) from May through November of 2006. Two white Leghorn chickens were housed in a 1.8m X 0.9m cage. The cage

was located in a field 100 m west of a horse pasture (Figure 4-1). It was placed next to a horse stall that was used as a stable trap (Chapter 3). The chickens were bled weekly from the brachial vein. Collections were taken from alternate wings each time the chicken was bled in order to allow healing. One ml of blood was collected in a gel separator vial with a 25-gauge needle and was centrifuged for 10 min at 600 g. The resulting sera were delivered to the Alachua County Health Department the same day they were collected. The samples were shipped with other Alachua County sentinel chicken blood samples to the Florida Department of Health Bureau of Laboratories in Tampa, where they were tested for Flavivirus and Alphavirus hemagglutination inhibition (HI) antibodies. The Department of Health routinely tests any resulting positive serum samples to identify WNV, SLEV, EEEV, or HJ antibody by IgM enzyme immunoassays and plaque reduction neutralization tests. The Alachua County health department received a weekly report of the results of the chicken serum tests, from the FDOH for all chickens tested in the County.

Mosquito Collections

Mosquito collections at the study site began on October 6, 2004 and continued to November 30, 2006. The traps were operated to two times a week (Figure 4-1) at 1600 hr and picked up the following morning at 0800 hr. Centers for Disease Control light traps (John W. Hock, Gainesville, Fl) baited with approximately 1 kg of dry ice were placed in four different habitats at the site: at the edge of Bivens Arm Park, next to the retention pond, under a small stand of oak trees, and by the horse stable trap (Figure 4-1). To analyze trap catch differences in four microhabitats, a Kruskal-Wallis one-way analysis of variance was used (Minitab version 15, State College, PA). PROC GLIMMIX analysis was done to compare the trap location microhabitats by month and to evaluate mosquito species collection between the two years of the study (SAS version 9.1, Cary, NC).

Mosquito collections were sorted by species and date of collection and stored in pools of up to 50 at -70°C. Pools were homogenized in 1 ml diluent of Phosphate Buffered Saline (PBS) with 4% Fetal Bovine Serum (FBS) by placing two copper BBs in the vial and vortexing. After a 10 min centrifugation at 11,356g to separate the mosquito solids, 200 µl of supernatant was transferred to a new tube for RNA extraction with Trizol® following the manufacture's protocol (Molecular Research Center Inc., Cincinnati, OH). The remaining sample, not used in the RNA extraction, was stored at –70 °C for possible cell culture if a positive result in RT-PCR was found. The RNA isolated with an RNeasy mini kit (QIAGEN, Valencia, CA) from the mosquito pools, and was tested for WNV, SLEV, and EEEV using quantitative Real Time RT-PCR (Lanciotti and Kerst 2001, Stark and Kanzanis 2007).

Four hundred microliters of the RNA extraction homogenate, and 600 µl L15 media (5% FBS, 15 µg/mL gentomyacin, 200 units/mL penicillin, streptomycin, fungizone) was added to a T25 cm^2 flask of Vero cells (2.0 x 10^6 cells). The cells were rocked and incubated at 37° C for 1h. Four mL L15 media was added and the cells were observed every 24h for 7d.

Meteorological Data

In order to address the relationship between weather and mosquito population dynamics, daily weather conditions including rainfall, wind speed, and average temperatures were accessed from the Florida Automated Weather Network (FAWN) recording station in Gainesville, FL (29 39'N, 82 30'W). A 50-year mean of precipitation and temperature was calculated from 1951-2000 by compiling data from the National Oceanic and Atmospheric Association (NOAA) archives. Monthly deviations from normal for precipitation and temperature were calculated by subtracting recorded values from the 50-year mean monthly values. Paired t-tests were used to evaluate differences between the two trapping season's temperature, precipitation, and vector

abundance. Linear regression was used to examine independent relationships between average mosquito catch per trap night and weekly average precipitation, temperature, and wind speed.

Results

Sentinel Animals

None of the five sentinel animals (two horses, one pony, and two sentinel chickens) maintained at the study site seroconverted to an arboviral agent during the study period. During the same time period, the sentinel chickens maintained by Alachua County Public Health Unit showed the following seroconversion activity: 2005, 16 WNV, 47 EEEV, and 3 HJ; 2006, 0 WNV, 15 EEEV, and 1 HJ (Table 4-1). A confirmed arboviral infection in a Florida horse is classified as a reportable disease in which case the attending veterinarian must report the case to the Florida Department of Agriculture and Consumer Services in Tallahassee. Between May 1 and August 29 2005, nine horses in Alachua County were confirmed as EEEV positive. One horse was positive for WNV; this report came on October 20, 2005. In 2006, no WNV horse cases were reported in Alachua County. Only a single EEEV horse case was reported on July 31 in Alachua County in 2006 (Table 4-1).

Mosquito Collections

Three hundred fifty nine mosquito pools (n = 13,809 total mosquitoes; 7 species) were tested for WNV, EEEV, and SLEV. One pool of 50 *Ma. tttillans,* collected in September 26, 2006 was positive for SLEV (minimum infection rate of 0.254). The attempt to grow the SLEV in Vero cell culture was unsuccessful. After 7d of observation no cytopathology was observed in the cells. The mosquito pools were collected over a period of 26 months (October 2004 through November 2006). During the same time period, Alachua County did not submit mosquito pools to the FDOH for testing. In the state of Florida in 2005 there were 1,603 mosquito pools tested from 11 counties. Five mosquito pools from three Florida counties (Monroe, Pinellas, and

Sarasota) tested positive for WNV and ten mosquito pools from four Florida counties (Escambia, Sarasota, St. Johns, and Volusia) tested positive for EEEV. In 2006, there were no positive mosquito pools (n = 1,253) in the state of Florida.

The most frequently collected mosquito species at the study site was *Mansonia titillans* (29%) followed by *Culex nigripalpus* (25%) (Figure 4-2). The seasonal distribution of the seven most abundant species was compared with horse and chicken seroconversions for WNV and EEEV in Alachua County (Figures 4-5 to 4-8). In 2005, the abundance of *Cx. erraticus* increased in February and March and the first EEEV chicken seroconversion was reported in April. In 2006, *Cx. erraticus* abundance increased in May and the first EEEV chicken serocnversion was at the end of May (Figures 4-6 and 4-8). The number of *Ma. titillans* increased in the fall, which was when the last of the chicken seroconversions and horse cases in both 2005 and 2006 were seen.

When trap location was examined by Kruskal-Wallis and Proc Glimmix analysis, the highest numbers of mosquitoes were trapped in the Bivens Arm Forest and the lowest numbers were obtained by the horse stable (Table 4-2, Figure 4-4). The collection of *Anopheles crucians* (P = 0.001), *Cx. erraticus* (P = 0.045), and *Ps. columbiae* (P = 0.04) varied significantly by location. There was no significant difference in the number of *Ma. titillans*, *Cx. nigripalpus, Cx. quinquefasciatus,* and *Oc. infirmatus* caught at each trap location. During each month of the study, the trap located in the Bivens Arm Forest collected significantly more mosquitoes that traps number 2 and 3. No significant difference in trap catch were seen between trap 1 (Bivens Arm) and trap 4 (the retention pond) in November and December, but all other months trap 1 caught significantly more mosquitoes than trap 4 (Figure 4-4). Trap year (year 1, October 2004 to October 2005 and year 2, November 2005 to November 2006) was significantly different for

collections of *An. quadrimaculatus*, *Cq. perturbans*, *Cx. nigripalpus, Cx. salinarius,* and *Oc. infirmatus*.

Meteorological Data

December 2005 was abnormally wet, with 48.6 cm of rainfall above the expected 50 yr (1951-2000) mean for Alachua County (Figure 4-3A). During the winter months of January through March 2005, the observed monthly rainfall amounts were 3 to 5 cm below average (Figure 4-3A). In 2006, the winter months of January through March were unusually wet, accumulating over 20 cm of rainfall above normal. In March 2006, a drought began and continued through September. During 2006, North Florida experienced a prolonged drought that resulted in a total of 15.4 cm less rainfall than average (Figure 4-3A). The mean daily rainfall patterns were significantly different between 2005 and 2006 (2005 mean 5.00 mm, 2006 mean 2.77 mm, $t_{(364)} = 1.66$, $P = 0.048$).

The temperatures ranged from 1 to 3 °C cooler than average for most of the study period (Figure 4-3B). Each month was colder than expected when compared to the long term means except for January of each year and April of 2006. The mean monthly temperatures were not significantly different between 2005 and 2006 (2005 mean 66.28° C, 2006 mean 66.23° C, $t_{(11)} = 0.064$, $P = 0.47$). There were no significant correlations of temperature, wind speed, or rainfall with mosquito abundance.

Discussion

Although arbovirus (WNV, SLEV, and EEEV) activity was minimal in Florida during the years of this study, information gathered during inter-epidemic years is valuable to the complete understanding of mosquito-borne disease epidemiology (Hay et al. 2000). Virus transmission is dependent on the presence of an abundant and old mosquito population (Zyzak et al. 2002) and mosquito reproduction and mortality are directly influenced by meteorological conditions.

Weather conditions including temperature and rainfall directly affect vector population distribution and abundance (Hay et al. 2000). During the spring of 2006 there was a prolonged drought in north Florida that reduced the abundance of mosquitoes and minimized arbovirus transmission (FDOH 2007).

The year-to-year differences in mosquito abundance and diversity at the study site are likely related to local weather patterns. The weather was wetter than average in north Florida in March through June and in October through December of 2005. Conversely, in 2006 there was a prolonged drought in Florida and very little arboviral activity was reported throughout the state. At the study site, some mosquito species (*Cx. nigripalpus* P = 0.0015, *Cx. salinarius* P = 0.0003, *An. quadrimaculatus* P = 0.0002) were collected in significantly fewer numbers in 2006 when compared to 2005. Transmission of SLEV and WNV are closely associated with rainfall patterns (Day 2001, Shaman et al 2005). The data presented here support the conclusion of drier years reducing the number of potentially infective mosquitoes (Day and Lewis 1991). The total range of mosquito habitat is dependent upon the presence of available bodies of water and humid daytime resting habitats. Therefore, a reduction in the number of infective mosquitoes decreases the likelihood of arbovirus transmission.

The trap located in Bivens Arm Forest (trap 1) collected significantly more *An. crucians, Cx. erraticus* and *Ps. columbiae* than the other three trap locations. Sylvan microhabitats may support a greater population of mosquitoes because they provide a daytime resting habitat and retain a high humidity. Additionally, the aquatic environment of Bivens Arm Lake provided larval habitat. Animals located near such a habitat may experience a greater number of mosquito bites by these species. The more open habitats of the oak stand, the stable, and the retention pond had significantly fewer mosquitoes collected and this is likely because these areas did not

retain high daytime humidity levels to support daytime resting. Therefore, the mosquitoes would need to fly much further from the daytime resting habitat of the forest to encounter a host in these open habitats.

There were no significant differences in the number of numbers of *Cx. nigripalpus, Ma. titillans,* and *Cx. quinquefasciatus* collected at each trap location. Two of the most abundant species collected at the study site, *Ma. titillans* and *Cx. nigripalpus*, were collected equally at all four trap locations. Day et al. (1991) found parous *Cx. nigripalpus* females collected in abundance in open habitats. They reported that abundance was especially high during wet summers when normally dry habitat became moist and humid allowing mosquitoes access to hosts. When surveying the flight capacity of blood-engorged mosquitoes, Edman and Bidlingmayer (1969), found *Cx. nigripalpus* in higher numbers in wooded habitats compared to open habitats. In my study, the fact that no significant difference of *Cx. nigripalpus* abundance occurred in the four trap microhabitats is consistent with the literature that this species can be collected in high numbers in either open or wooded habitats. The ubiquitous nature of *Cx. nigripalpus* at the study site may increase the likelihood of a single mosquito encountering both a reservoir host and a susceptible host. *Mansonia titillans* was abundant at this site in the late fall and was not significantly correlated with trap location. The lake at Bivens Arm contains abundant aquatic flora (waterhyacinth, *Eichhornia crassipes* (Mart.) Solms., and waterlettuce, *Pistia stratiotes* L.) necessary for the larval development of *Ma. titillans* populations. In a dispersal study of several *Mansonia* species, individuals were re-captured from 0.5 to 2.4 km from the release point (Macdonald et al. 1990). This flight distance is adequate to explain why approximately equal collections of *Ma. titillans* were obtained in all the habitats surveyed.

Location of sentinel chicken sites is an important factor when initiating an arbovirus surveillance program (Day et al. 1991). The historical enzootic activity of WNV at this site was a key factor for choosing to place sentinel chickens there. None of the sentinel animals (three equines and two chickens) at the site was positive for WNV, SLEV, HJ, or EEEV during this study. Although the sample size was small with a total of five animals being screened, there were no WNV seroconversions of sentinel chickens in Alachua County in 2006 (Table 4-1). Moreover there were no horse or human WNV cases in Alachua County in 2006. Because sentinel animals in Florida provide the most accurate and timely indication of field transmission, the lack of seroconversions at our site may be an indication of low-level virus circulation in the area in 2005 and 2006.

Although collected in large numbers, *Ma. titillans* did not show a temporal correlation in abundance with the EEEV or WNV transmission season in Alachua County in 2005 and 2006 (Figure 4-4). West Nile virus has been isolated from field caught *Ma. titillans* (CDC 2007), but no work has been published regarding their vector competence (Turell et al. 2005). Vector capacity is determined not only from natural infection and demonstrated laboratory transmission, but also biological factors such as biting preference, length of life, and timing of adult activity are all fundamentally tied to vector capacity (DeFoliart et al 1987). Because *Ma. titillans* did not appear at the study site until EEEV and WNV transmission had already begun in Alachua County in 2005 it may not play a major vector role in arbovirus epidemics in north Florida. Despite the lack of temporal correlation with EEEV and WNV transmission at the site, more research is likely warranted as *Ma. titillans* is considered a vector of Venezuelan Equine Encephalitis virus (VEEV; Family *Togaviridae,* genus *alphavirus*) in Central and South America (Mendez et al. 2001, Turell et al. 2000). *Mansonia dyari* may be a maintenance vector of SLEV

in Panama (Gorgas Memorial Laboratory 1979, as cited by Lounibos et al. 1990). Members of the genus *Mansonia* in Africa have had several WNV isolations reported (Traore-Lamizana et al. 2001). Furthermore, several species of *Mansonia* are likely involved in the transmission of Japanese Encephalitis virus (JEV; family *Flaviviridae,* genus *Flavivirus,*) in Asia (Arunachalam et al. 2004). This is the first positive identification of SLEV from *Ma. titillans* in Florida. The failure to isolate virus in cell culture may have been because no cryogenic protection (i.e. DMSO) was added to the homogenate. Freezing and thawing the sample reduces viable virions because ice crystals break the envelope. Another potential reason virus was not isolated is that the mosquito pools may have been stored at -20° C for a period of time and research indicates that -70° C is the optimal storage temperature for virus detection of mosquito pools (Turell et al. 2002).

Culex erraticus populations increased at the study site a few weeks to a month prior to EEEV transmission in Alachua County. In Alabama EEEV isolations from field caught *Cx. erraticus* have been reported (Cupp et al. 2003). The natural isolations in the southeastern United States and temporal correlation may indicate that this species is involved in EEEV transmission in north Florida. *Culex nigripalpus* was active at low levels throughout the duration of the study. In 2005 in Florida, there were three WNV isolations from *Cx. nigripalpus* (2 in Pinellas and 1 in Sarasota County) and transmission has been documented in Jefferson County, Florida (Rutledge et al. 2003). However, at our study site the population of *Cx. nigripalpus* after November 2004 remained low (n < 50/trap night) for the duration of the study. Perhaps the low numbers were related to weather conditions unfavorable for mosquito development.

Having only four light traps in different locations at the site was a limitation of the study. A better assessment of microhabitat could have been made if the traps were moved within a

single microhabitat randomly to exclude the possibility of a trapping-out effect. A second limitation of the study was the fact that only five sentinel animals were screened for arbovirus activity. If more animals were used, there would have been a better chance of observing virus activity had there been any. A comparison of arboviral activity in Alachua County with the mosquitoes present at the study site was possible because the county maintains sentinel flocks and horse cases are recorded. However, in this study, mosquitoes were collected in one place and transmission occurred in another, making interpretation more difficult. The seasonal abundance of *Cx. erraticus* and *Cx. nigripalpus* at this site increased prior to EEEV and WNV transmission in Alachua County. These two species should be further considered as potential vectors in north Florida of EEEV and WNV respectively.

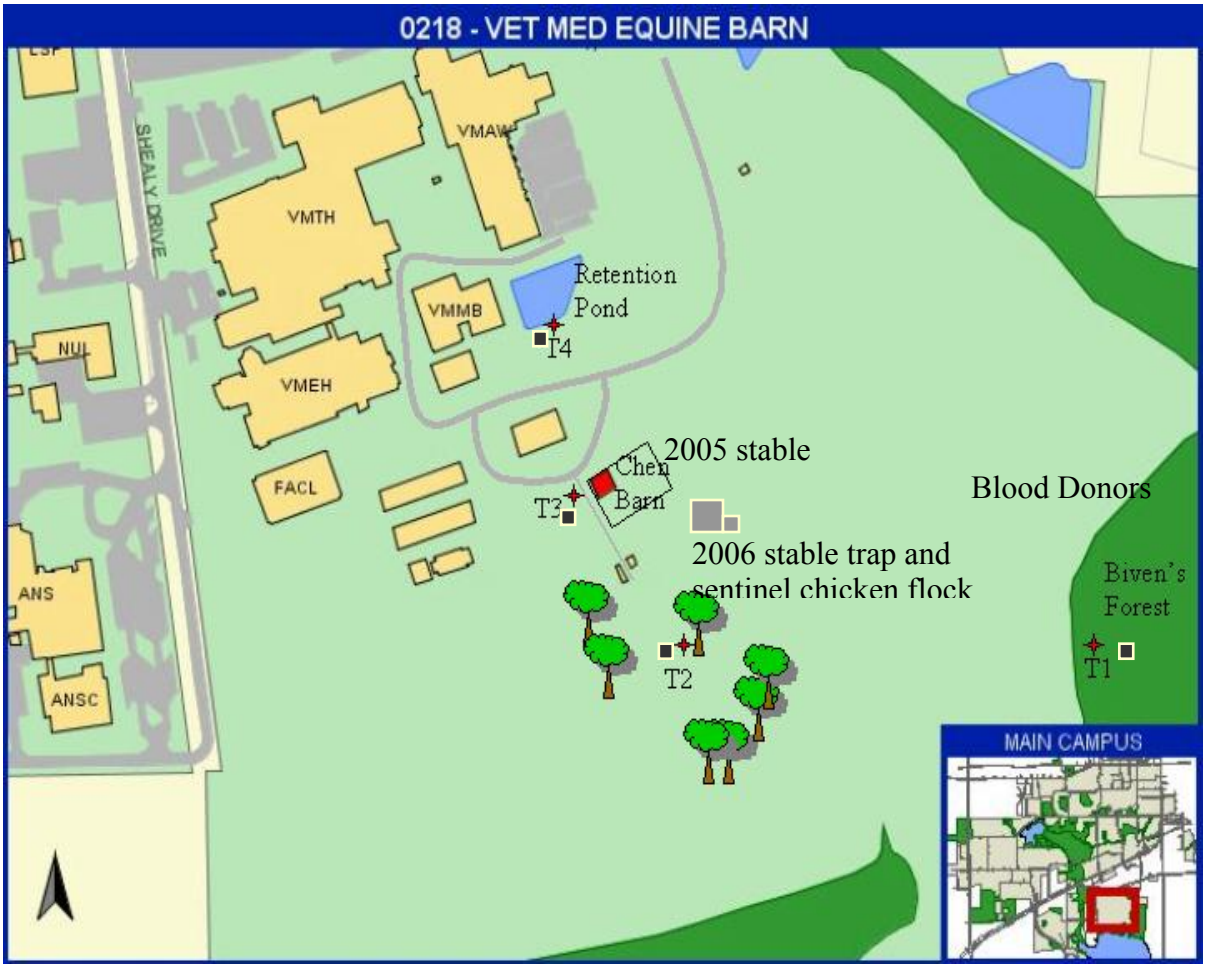


Figure 4-1. Location of the four paired light traps and resting boxes marked as T1, T2, T3, and T4. The Chen barn was modified for the stable trap in 2005, and a portable stall was erected 14m south of the Chen barn during the 2006 season.

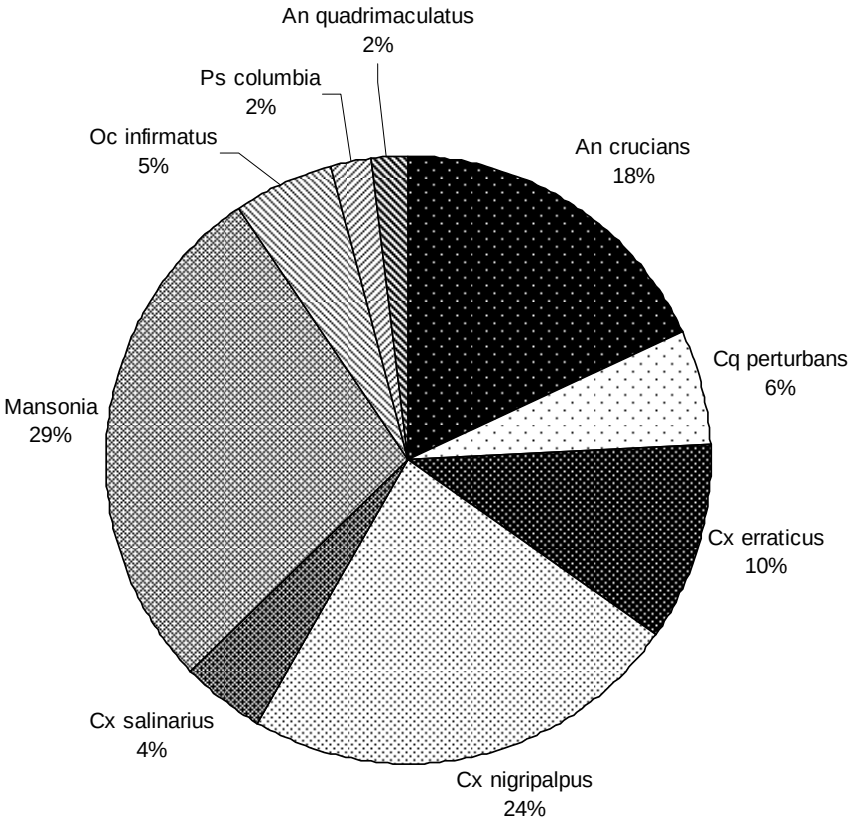


Figure 4-2. The species that represented at least 1% or more of the total trap catch between October 2004 and November 2006

Table 4-1. Number of arbovirus positive sentinel chickens and horses in Alachua County in 2005 and 2006.

Arbovirus	Chicken 2005	Horse 2005	Chicken 2006	Horse 2006	Total
EEE	47	9	15	1	72
WNV	16	1	0	0	17
SLE	0	0	0	0	0
HJ	3	0	1	0	4
Total	66	10	16	1	93

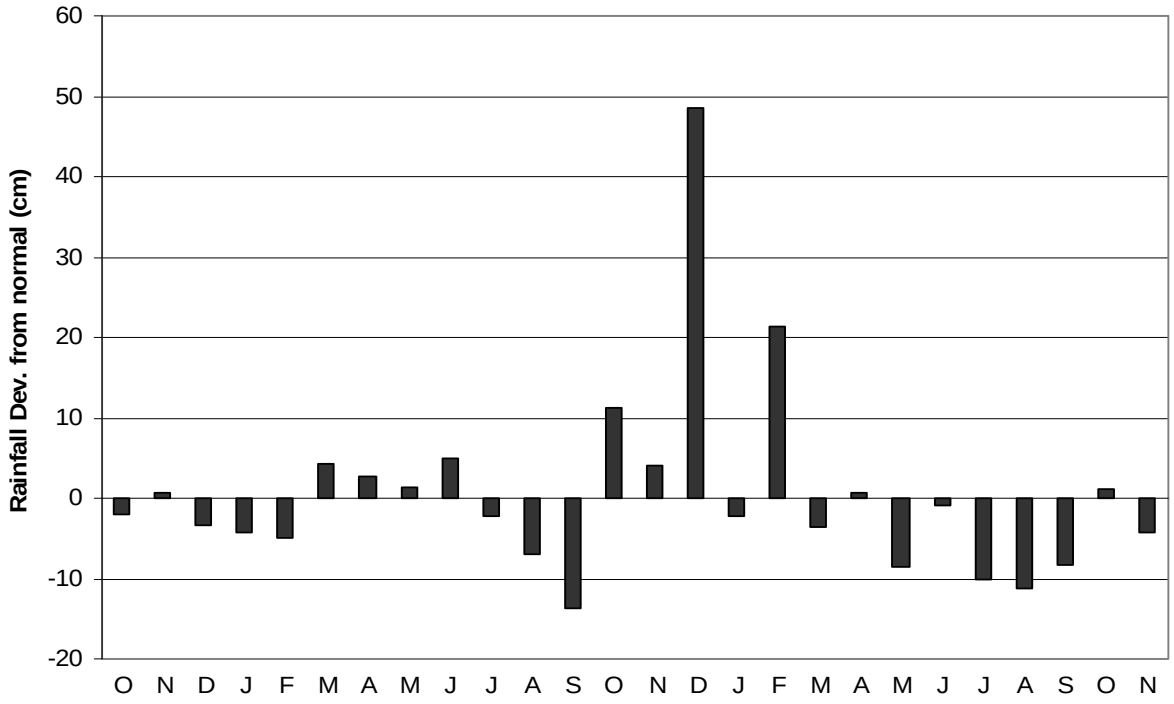
Table 4-2. Mosquito collections by trap location from October 2004 though October 2005 (13 months). Mosquitoes were collected from light trap collections..

Light traps (Oct 2004-Oct 2005)	Location				
Species	Woods	Pond	Oak trees	Stable	Total
Cx. salinarius	928	219	296	55	1498
Cx. nigripalpus	7060	1441	1952	77	10530
Cx. erraticus	1291	136	101	44	1572
Oc. infirmatus	1753	281	50	10	2094
An. crucians	2377	461	117	36	2991
An. quadrimaculatus	444	120	36	19	619
Mansonia titillans	1851	1819	527	233	4430
Total	15704	4477	3079	474	23734

Table 4-3. Mosquito collections by trap location from November 2005 though November 2006 (13 months). Mosquitoes were collected from light trap collections.

Light traps (Nov 2005-Nov 2006)	Location				
Species	Woods	Pond	Oak trees	Stable	Total
Cx. salinarius	339	109	31	21	500
Cx. nigripalpus	112	14	6	3	135
Cx. erraticus	2455	206	224	217	3102
Oc. infirmatus	273	48	3	1	325
An. crucians	4939	141	55	23	5158
An. quadrimaculatus	236	14	32	4	286
Mansonia titillans	2845	2262	2194	228	7529
Total	11199	2794	2545	497	17035

A



B

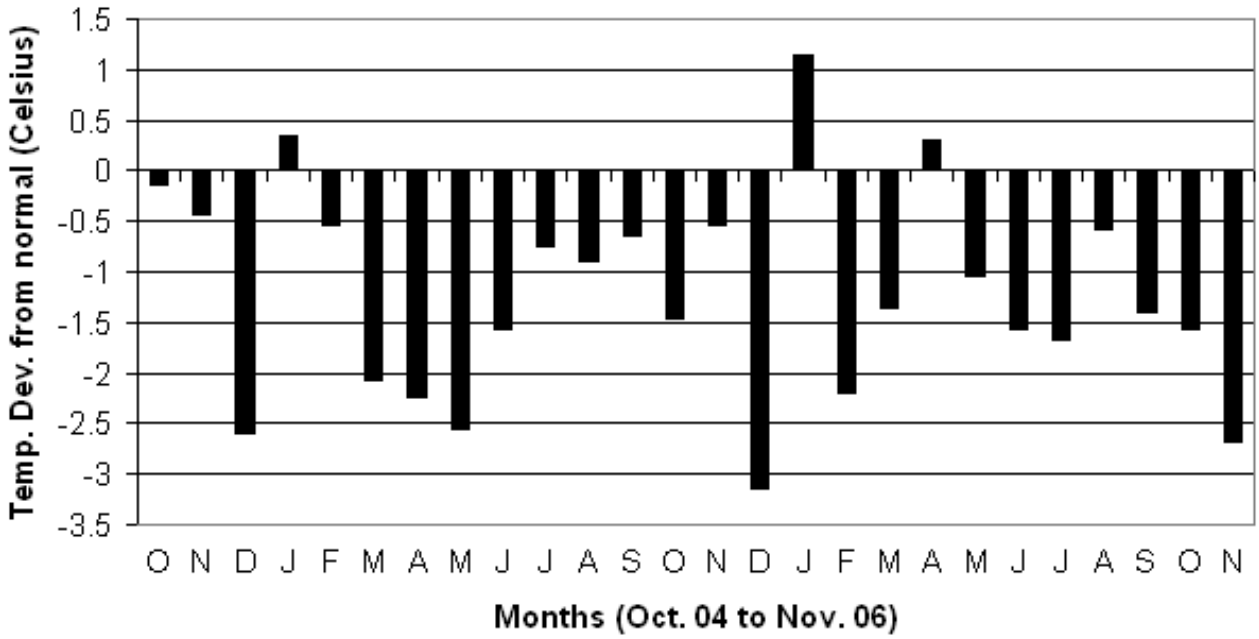


Figure 4-3. Monthly deviations from normal for rainfall A) and temperature B) for October 2004 through November 2006 in Alachua County Florida. Observed monthly values were subtracted from a 50-year mean (1951-2000) to determine the deviations from normal.

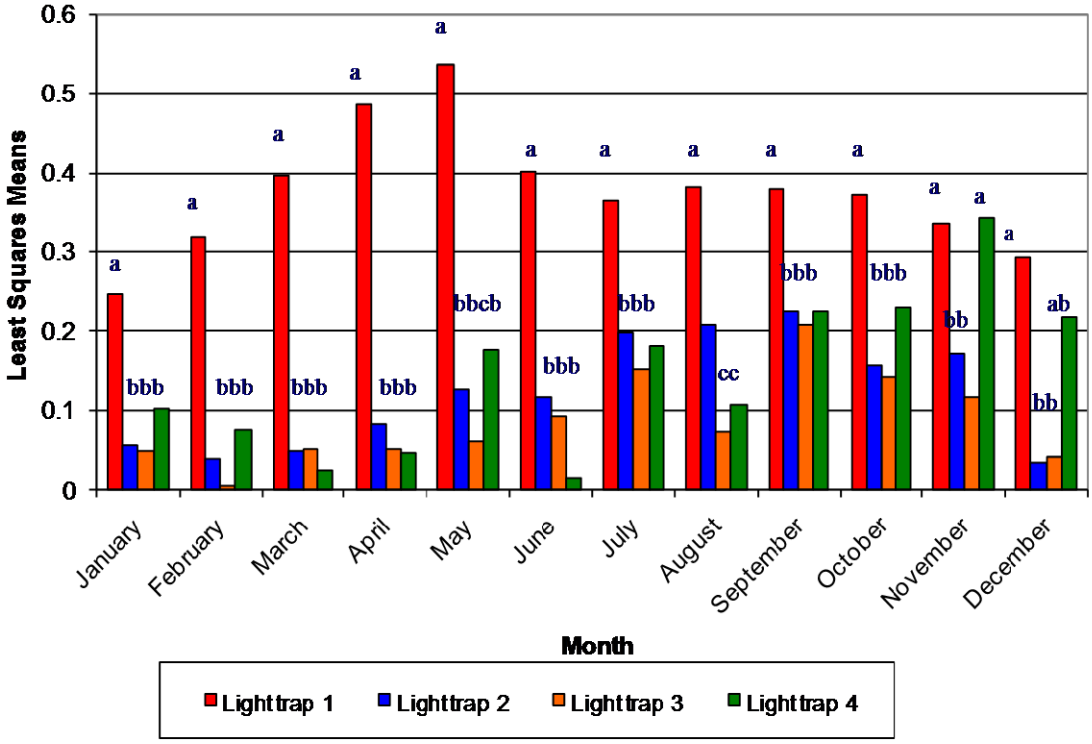


Figure 4-4. Comparison by month of the four light trap locations average mosquito trap catch. Bars followed by a different letter are significant at $P < 0.05$.

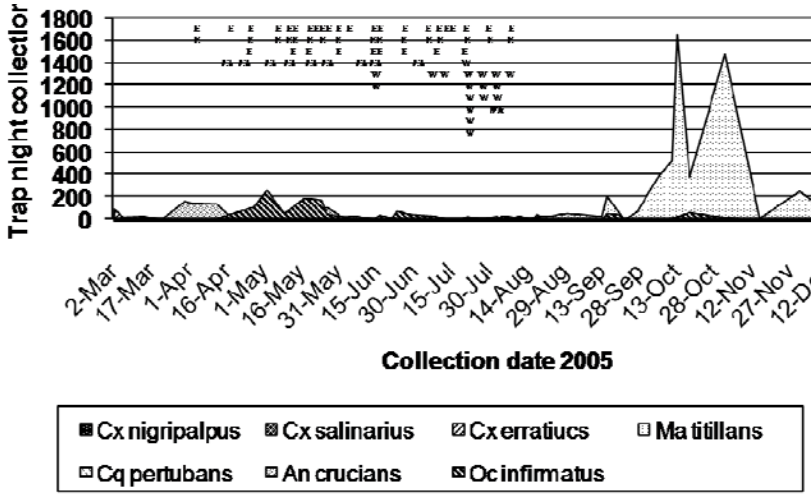


Figure 4-5. Temporal distribution of the seven most abundant mosquito species collected at the University of Florida Veterinary School (March through December 2005). E, Eastern Equine Encephalitis in Alachua County sentinel chickens (*n* = 47); *Eh*, Eastern Equine Encephalitis in Alachua County horses (n = 9); W, West Nile virus in Alachua County sentinel chickens (*n* = 16); *Wh*, West Nile virus in Alachua County horse (*n* = 1).

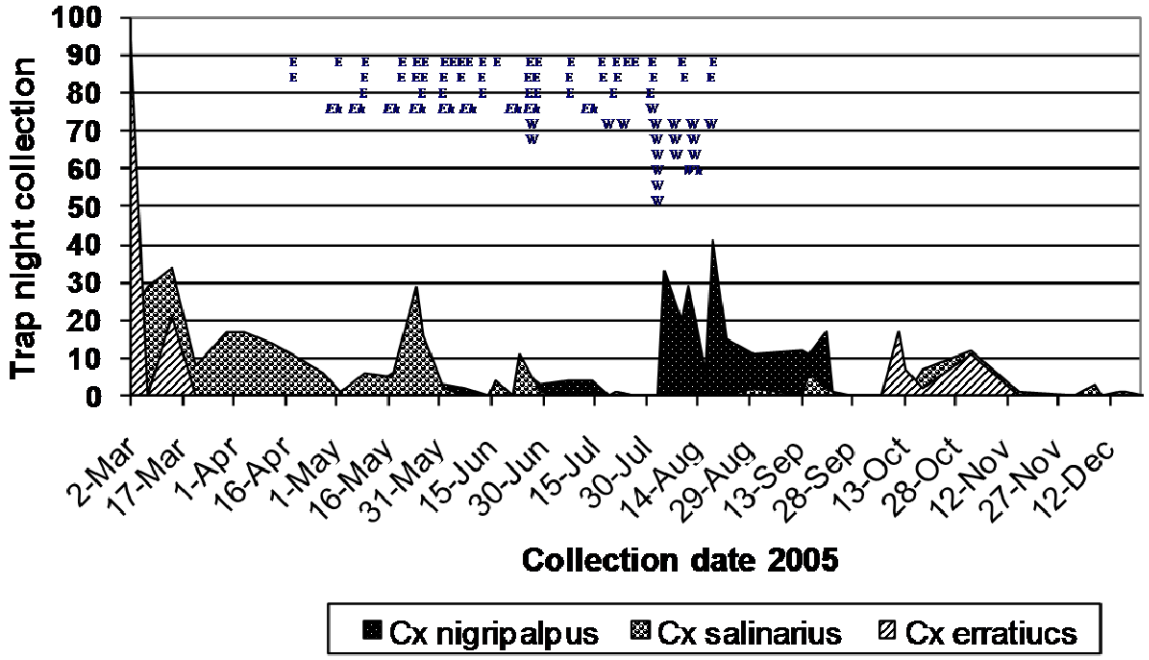


Figure 4-6. Temporal distribution of three *Culex* mosquito species collected at the University of Florida Veterinary School (March through December 2005). E, Eastern Equine Encephalitis in Alachua County sentinel chickens (*n* = 47); *Eh*, Eastern Equine Encephalitis in Alachua County horses (n = 9); W, West Nile virus in Alachua County sentinel chickens (*n* = 16); *Wh*, West Nile virus in Alachua County horse (*n* = 1).

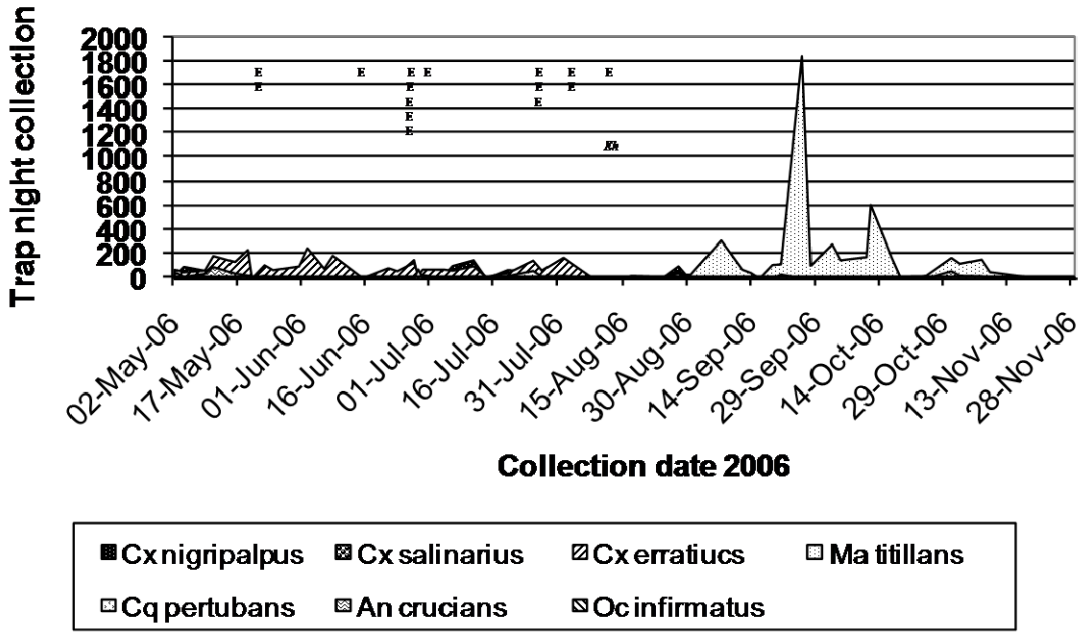


Figure 4-7. Temporal distribution of seven most abundant mosquito species collected at the University of Florida Veterinary School (May through November 2006). E, Eastern Equine Encephalitis in Alachua County sentinel chickens (n = 15); *Eh*, Eastern Equine Encephalitis in an Alachua County horse (n = 1).

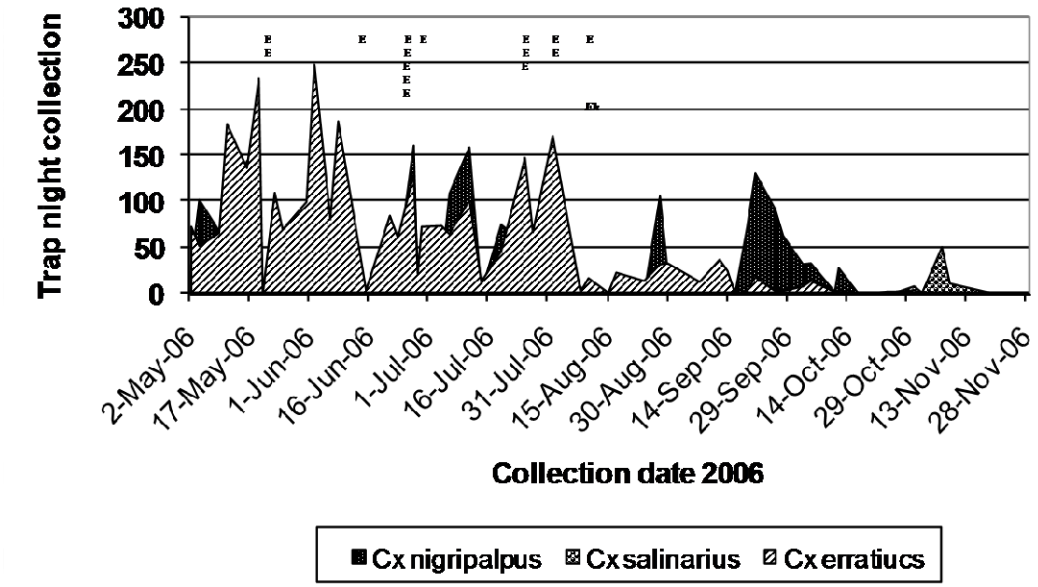


Figure 4-7. Temporal distribution of three *Culex* mosquito species collected at the University of Florida Veterinary School (May through November 2006). E, Eastern Equine Encephalitis in Alachua County sentinel chickens (n = 15); *Eh*, Eastern Equine Encephalitis in an Alachua County horse (n = 1).

CHAPTER 5
BLOOD MEAL IDENTIFICATION OF MOSQUITOES COLLECTED FROM LIGHT TRAPS IN NORTH CENTRAL FLORIDA (2004-2006).

The dynamics of vector and host interactions are an integral part of understanding disease transmission. Knowledge of vector host feeding patterns provides insight to viral transmission cycles by identifying possible host preferences. Techniques in blood meal analysis have been changing since the early 1920s and have included direct observation of feeding mosquitoes, quantification by capture in host-baited traps, and serological and genetic based techniques (Ngo and Kramer 2003). The most common serological and genetic based techniques have been the precipitin test, the Enzyme Linked ImmunoSorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) assays (Ngo and Kramer 2003). Polymerase Chain Reaction amplification of host DNA followed by sequencing is becoming a common method for blood meal detection and has several advantages over precipitin tests and ELISA. Due to the sensitivity of the PCR, a very small amount of DNA can be used as template so even partially engorged mosquitoes can yield a blood meal confirmation. Additionally, with serological tests such as precipitin and ELISA, anti-sera must be prepared for each potential host species allowing blood meal confirmation to only a limited number of species. With the advent of web-based databases such as GenBank, it is now possible to compare nucleotide sequences and identify sources of arthropod blood meals.

Many studies of mosquito blood meals have emphasized the avian host identifications (Lee et al. 2002; Ngo and Kramer 2004). Other studies have focused on mosquito feeding patterns by distinguishing between avian and mammalian derived blood meals (Apperson et al. 2002). Mosquitoes that are primarily ornithophilic, such as *Culiseta melenura* (Coquillett) for eastern equine encephalitis virus (family *Togaviridae,* genus *Alphavirus,* EEEV) (Scott et al. 1984), play a major role in the amplification of arboviruses. Some mosquito species in the genus *Culex* prefer avian hosts and are important enzootic vectors of St. Louis encephalitis virus (family

Flaviviridae, genus *Flavivirus,* SLEV) (Tsai and Mitchell 1989). *Culex nigripalpus* Theobald is an opportunistic feeder and may serve as an important vector of SLEV and West Nile virus (family *Flaviviridae,* genus *Flavivirus,* WNV) in Florida (Day 2001). Additionally, *Culex salinarius* Theobald may play a secondary role in the transmission of both SLEV and WNV during times of the year *Cx. nigripalpus* is less abundant (Day 2001). Another important factor for epidemic arboviral transmission is the well-documented host switching behavior of *Cx. nigripalpus* (Edman 1974). *Culex nigripalpus* feeds preferentially on birds in the winter and spring and shows an increased preference for mammalian hosts in the later part of the summer and into the fall.

Entomological measures of arboviral transmission risk can be estimated by considering mosquito abundance and age, biting preference, field isolations of virus, and vector competence (Molaei et al. 2006). The primary *Culex* mosquito vector of WNV differs by geographic location (Hayes and Gubler 2006). Research in Florida suggests that *Culex nigripalpus* is an important enzootic and epidemic vector of WNV to humans (Rutledge et al. 2003, Shaman et al. 2005). However, research is lacking on which mosquito species may potentially transmit WNV to horses in Florida.

Horses are susceptible to WNV infection and each year fatal WNV horse cases are reported in the US. The purpose of this study was to determine the mosquito blood feeding patterns at a site where horses were stabled outdoors year round. To accomplish this goal a two-year project was designed to collect blood fed mosquitoes located near horses. The results of this study may provide valuable insight to potential mosquito species vectoring WNV to horses in north Florida.

Materials Methods

Blood Fed Mosquito Collections

All blood fed mosquito collections were made at a site (29 38' N, 82 20' W) in Alachua County in North Florida. Horses (n = 50) were maintained in outdoor pastures and in the spring, when mares delivered, the total number of horses on the property increased to approximately 70. The site was an open pastureland surrounded by urban development (Chapter 3 Materials and Methods). Mosquitoes were collected twice a week using four CDC light traps and a backpack aspirator (John Hock Co., Gainesville, FL) (Chapter 3 Materials and Methods). Mosquitoes were aspirated from four resting boxes that were paired with a light trap and from the outside of the horse-baited stable trap. Additional resting collections were taken from 10-minute ground aspirations in the surrounding vegetation next to light trap number 1 (Figure 3-1). Light trap number 1 was located within the sheltered habitat of trees, along the edge of dense vegetation and open pasture where horses were always present. Light trap number 2 was located under a small oak stand consisting of seven mature trees. Vegetation and under story at this trap site was sparse and standing water was present after a rainfall event. Light trap number 3 was placed adjacent to a horse stable. The grass surrounding the stable was mowed short and no trees were located in the vicinity. Light trap number 4 was set next to a 1500 sq ft retention pond with aquatic vegetation including cattails (*Typha latifolia* L.*)*, rushes (Family *Juncaceae*), and waterlettuce (*Pistia stratiotes* L.) (Figure 3-1). All mosquitoes taken from aspirator and light trap collections were immediately transported to the laboratory, where they were stored at -80˚C. They were then counted on a chill table and identified and sorted by species according to Darsie and Morris (2003).

All blood engorged females were separated from the collection and stored individually at -80°C. Each mosquito was identified to species and the size and stage of the bloodmeal were

recorded. The size of the bloodmeal was categorized according to Edman et al. (1975), the sizes were: trace (no distention of the abdomen), 1/4, 1/2, 3/4 , and 1 (fully fed). The stage (estimation of days after a blood meal based on appearance of the abdomen) of the bloodmeal was categorized according to Sella (Detinova 1962) on a one to seven scale, one (unfed) to seven (fully gravid) scale.

Blood Meal Identification.

The extraction and PCR procedures were validated and optimized with positive controls. The mosquitoes used as positive controls were *Cx. quinquefasciatus* and *Cx. nigripalpus* that were obtained from the USDA (United States Department of Agriculture CMAVE), Gainesville colonies. These mosquitoes were starved for 24 h prior to blood feeding. *Culex quinquefasciatus* was fed cow blood from a sausage lining and *Cx. nigripalpus* was allowed to feed on a live chicken (feeding that is a normal part of colony maintencance at the USDA). The engorged female mosquitoes were frozen at - 20° C. After it was validated that the primer sets were successfully amplifying the target DNA, the remaining aliquot from the known host DNA extaction was used as the positive control in subsequent PCRs. An unengorged female mosquito was used as the negative control to ensure that no invertebrate DNA was amplified in the PCR.

Blood fed mosquitoes were thawed and placed on a Kimwipe® tissue. The abdomen was separated from the thorax by using sterile pipette tips to sever the tegument and isolate the bloodmeal from all extraneous material. The DNA from the mosquito abdomen was isolated using a phenol chloroform extraction (Levy et al. 2002). The DNA pellet was resuspended in 10 µl of 1mM Tris, and stored at -70 °C until later analysis.

Two separate PCR reactions were used to amplify the DNA template. Each PCR contained a distinct primer set from the cytochrome b region of the mitochondrial genome.

Primers were chosen based on previously published blood meal analyses, one primer set was designed to amplify avian blood (Cicero and Johnson 2001) and the other primer set was designed to amplify mammalian blood (Ngo and Kramer 2003) (Table 5-1). A third set of vertabrate specific primers was used to amplify the sequences if results could not be obtained from the first PCR attempt (Cupp et al. 2004) (Table 5-1).

Mammalian and avian PCR assays were run in a final volume of 25 µl. Each reaction contained 0.5 mM dNTPs, 3mM $MgCl_2$ and 1.2 units of Taq polymerase (Invitrogen, Carlsbad, CA). The avian assay contained a final primer concentration of 15 pmole per reaction and the mammalian assay contianed 5 pmole of each primer per reaction. Amplification conditions for the avian PCR were 5 min at 93˚C with 45 cycles of 94˚C for 30 s, 50˚C for 30 s, and 72˚C for 1 min 30 s with a final extension of 3 min at 72˚C.

The amplification cycle of the mammalian PCR was equalivant to the avian PCR conditions, with the exception of the melting temperature, which was lowered to 48˚C. The vertebrate specific assay conditions were 2 min at 94˚C with 55 cycles of 94˚C for 45 s, 50˚C for 50 s, and 72˚C for 1 min with a final extension of 7 min at 72˚C. The products were visualized on a 1% agarose gel stained with ethidium bromide under UV light.

The bands of expected size (508 bp for avian and 772 bp for mammalian) were cut from the gel and the DNA was extracted using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified DNA was sequenced using BigDye Terminator Kit version 1.1 (ABI prism, Foster City, CA). The Interdisciplinary Center for Biotechnology Research (ICBR) Sequencing Core at the University of Florida loaded the sequenced DNA and ran the product on a gel. The electropherograms (base pair sequence information) were edited using Sequencher version 4.1.2

software (Macintosh). The edited sequences were compared to the nucleotide database with BLAST analysis software available through NCBI.

Results

From a total of 45,326 mosquitoes collected in CDC light traps and resting collections, 242 (0.53%) were engorged. Of the 23 mosquito species identified at the study site, 14 species were blood fed. A confirmed host blood meal match was obtained for 143 samples of the 242 (59%) blood fed mosquitoes collected. The blood meals were identified to species or in some cases order and represented mammalian (95%, 136/143), reptilian (2%, 3/143), and avian (3%, 4/143) hosts (Table 5-2).

The results of the blood meal analysis are summarized in Table 5-2. Of the blood meals obtained from reptiles, the turtle blood meals were isolated from *Culex erraticus* and *Ochleratatus infirmatus* and the anole blood meal was isolated from *Culex nigripalpus*. A mixed blood meal was isolated from one *Aedes vexans* that had fed on both a human and a horse. With the avian primer set there was non-specific amplification of cow and reptilian hosts. The blood meals (n = 9) isolated from *Cx. salinarius* were from horse and human. The most common blood meal isolated from *Cx. nigripalpus* was horse (58%); the other blood meals were from human, raccoon, cow and anole. *Mansonia titillans* fed on horse, human, mouse, cow, and there was a single isolation from a chicken (Table 5-2).

Non-specific amplification with the avian primer set occurred. A PCR amplified DNA band of the correct size was obtained in both assays with the mammalian primers and the avian primers for two samples. Prior to sequencing this was thought to be a mixed blood meal, where the mosquito had partially engorged on two separate hosts. Upon sequencing, both matched up 100% with *Bos taurus*, the domestic cow. A control sample of cow blood was run with both primer sets and amplified in each assay. Additionally, the avian primer set amplified the two

reptilian derived blood meals. This primer set was designed to be avian specific, but non-target amplification did occur. However, the unfed mosquito used as a negative control never showed any amplification, and all the avian controls (chicken, dove, and vulture) amplified consistently. Based on the consistency of amplification in the positive controls of avian blood, and the absence of amplification of the mosquito negative controls, it appears that there was no interaction with these primers and mosquito DNA, and when avian blood was present, it amplified.

Discussion

Blood fed mosquitoes were collected in light traps, resting traps, and aspirator collections. Host identification studies usually focus on collection of mosquitoes from resting sites because these areas yield the highest number of blood fed individuals (Nasci 1984, Apperson et al. 2002). Therefore, the relatively large number of blood fed mosquitoes collected from light traps (n = 199) in this study was unexpected. Light traps specifically attract host-seeking females, and for this reason the majority of mosquitoes collected are normally unfed. However, Ngo and Kramer (2003) successfully used light traps in their study of blood meal identification. The light traps in this study likely collected most of the blood fed individuals because the available vegetation was inadequate to serve as a resting site. Most of the field site vegetation was located at the trap number 1, which was along the edge of the pasture. The vegetation was not as dense and probably not able to retain the high relative humidity characteristic of a suitable resting habitat (Day 2001).

To increase sample size all specimens containing any trace of blood were processed, regardless of Sella stage. A number of samples did not result in DNA amplification; the old age of the blood meal may have contributed to this. Studies of the time limit of blood meal detection have varied with reports of a maximum of 72 hours (Ngo and Kramer 2003) to a maximum of 7 days (Lee et al. 2002) before the blood meal was too far digested to amplify in PCR. The range

in detection limit is likely due to variation in individual mosquito species rate of blood digestion. Trace blood meals frequently amplified and the age of the blood meal, based on Sella stage, appeared to be a more important factor than size of the blood meal in this study. Blood meals at stage 6 only amplified 4 times and 16 blood meals did not. Because PCR is extremely sensitive, DNA extracted from a small blood meal, but not from an old blood meal, was adequate for amplification.

In this study there were only three avian blood meals amplified and three of these were chicken blood meals almost certainly obtained from the sentinel chickens being held adjacent to the stable trap. It is probable the height of the traps affected the diversity of mosquitoes caught. Traps placed in the tree canopy will capture higher numbers of mosquitoes that are likely feeding on avian hosts (Anderson et al. 2004). The trap height of 1 meter was chosen to target those mosquitoes active at the ground level and those most likely to feed on horses.

Horses were pastured in outdoor paddocks and were available to mosquito species active throughout the day and night. Horses were the most common host blood meal isolated in this study. It has been documented that *Cx. nigripalpus* is a widely opportunistic feeder and has a host range of birds, mammals, and reptiles (Day and Curtis 1994). Edman (1974) found a shift in the feeding pattern of *Cx. nigripalpus* from avian hosts in the spring to mammalian hosts in the fall. However, in this study *Cx. nigripalpus* only fed only on mammalian and reptilian blood. The sample size of 20 *Cx. nigripalpus* amplified blood meals was not sufficient to detect host switching at this site. The frequent (9/20) horse feeding by *Cx. nigripalpus*, found at this site along with previous research supporting the role of *Cx. nigripalpus* in WNV transmission, warrants further research into the importance of this species in WNV transmission cycles in north Florida (Rutledge et al. 2003; Shaman et al. 2005).

Culex salinarius has been implicated in transmission of SLEV and EEEV (Slaff 1990; Cupp et al. 2004). This species is documented as readily feeding on horses {Samui, 2003 2335 /id}, and has the ability to travel up to 2.0 km in 1.5 hr (LaSalle and Dakin 1982), allowing it to easily disperse out into the open pasture areas to feed. The blood meals (n = 9) from *Cx. salinarius* at this site were exclusively on horses and humans. *Culex salinarius* is considered a general feeder attacking indiscriminately both birds and mammals including humans (Andreadis et al. 2001). However, in open agricultural habitats *Cx. salinarius* has been documented feeding exclusively on mammals (Edman 1974). Because *Cx. salinarius* has had field isolations of WNV, is a competent laboratory vector of WNV (Turell et al. 2005) and feeds on avian and mammalian hosts it should be further studied in north Florida as a possible epidemic vector. Furthermore, *Cx. salinarius* is seasonally abundant in the early spring correlating with the amplification phase of WNV and is continually collected through June (Figures 4-6 and 4-8).

Mansonia titillans was the most frequently collected of the all the species represented at this site. From the 41 amplified blood meals from *Ma. titillans,* 26 were from horse. The remaining blood meals were from raccoon, mouse, cow, human, and chicken. The role of *Ma. titillans* in WNV transmission has not been well studied. But the fact that there was an avian isolation and it readily feeds on horses may be worth investigating further. The avian isolation, however, was from a chicken located at ground level and not an amplification host. Finally, the seasonal abundance of *Ma. titillans* does not correlate with amplification, or early transmission phases of WNV (Figures 4-5 and 4-7).

There were 53 human blood meals derived from this sample set isolated from eight mosquito species (Table 5-2). With students and workers outside or in open barns throughout

the day and evening, a host-seeking mosquito at this site would have frequent opportunity to encounter a human and obtain a blood meal.

As we examine the many epidemiological factors of disease transmission, the clues regarding host preference in wild caught mosquitoes will help broaden our understanding of vector-host interactions. By better understanding which mosquitoes play a key role in the transmission cycle, new control measures can be developed that are more efficient and target specific species. With tools such as DNA-based amplification, the host species are more readily and accurately able to be identified. The host preference information, when combined with the existing knowledge of mosquito biology, aids the effort to improve arbovirus surveillance and prevention strategies through control programs and public health advisories.

Table 5-1. Primer sets in PCR used to amplify DNA from vertebrate hosts.

Name	Sequence	Product size (bp)	Reference
Avian	5'-GACTGTGACAAAATCCCNTTCCA-3' 5'-GGTCTTCATCTYHGGYTTACAAGAC-3'	508	Cicero and Johnson (2001)
Mammalian	5'-CGAAGCTTGATATGAAAAACCATCGTTG-3' 5'-TGTAGTTRTCWGGGTCHCCTA-3'	772	Ngo and Kramer (2003)
Invertabrate	5'-CCCCTCAGAATGATATTTGTCCTCA-3' 5'-GCHGAYACHWVHHYHGCHTTYTCHTC-3'	228	Cupp et al. (2004)

H = A, C, or T; Y = C or T; V = A, C, or G; N =A, T, C, or G ; R = G or A ; W = A or T.

Table 5-2. Identification of blood meals from mosquitoes collected in Gainesville, FL, October 2004 to November 2006.

Mosquito Species	# tested	# confirmed (%)	Results
Ae. vexans	4	4 (100)	3 Human; 1 Mixed Horse and Human
An. crucians	4	4 (100)	3 Human; 1 Horse
An. quadrimaculatus	1	1 (100)	1 Horse
Cx. salinarius	14	9 (55)	4 Horse; 5 Human
Cx. erraticus	30	27 (90)	1 Night Heron; 1 Box Turtle; 6 Horse; 3 Raccoon; 16 Human
Cx. quinquefasciatus	3	1 (33)	1 Chicken
Cx. nigripalpus	36	20 (55)	1 Anole; 2 Cow; 9 Horse; 3 Raccoon; 5 Human
Ma. titillans	88	41 (56)	1 Chicken; 2 Mouse;3 Cow; 4 Raccoon; 5 Human; 26 Horse
Cq. perturbans	15	8 (53)	1 Armadillo; 1 Deer; 6 horse
Oc. infirmatus	9	7 (78)	1 Box Turtle; 2 Horse; 2 Human; 2 Raccoon
Oc. mitchellae	2	1 (50)	1 Horse
Ps. columbiae	28	19 (68)	1 Chicken; 1 Deer; 3 Horse; 14 Human
Ps. ciliata	3	1 (33)	1 Horse
Totals	237	143	

Horse (*Equus caballus*), Human (*Homo sapiens),* Raccoon (*Procyon lotor*), Cow (*Bos taurus)*, Chicken (*Gallus gallus*), Deer (*Odocoileus virginianus*), Mouse (*Mus musculus*), Night heron (*Nycticorax nycticorax*), Armadillo (*Dasypuis novemcintus*), Turtle (*Terrapene carolina*), and Anole (*Anolis trinitatis)*

CHAPTER 6
HOST FEEDING, VIRUS SURVEILLANCE AND FUTURE EXPERIMENTS

Summary

West Nile virus (Family *Flaviviridae,* genus *Flavivirus,* WNV) has become endemic in the US and the western hemisphere (Komar and Clark 2006). The introduction of WNV into the New World has provided a unique opportunity to study the spread and epidemiology of an arbovirus in a new geographic setting. The impact of WNV on horses and humans has facilitated collaborations between the veterinary and human medical fields. It has also sparked the development of new diagnostic and surveillance techniques that may help the United States prepare for future disease introductions. Many critical questions about potential mosquito vectors of WNV, the effects of microhabitat and weather on WNV amplification and transmission, and the blood feeding patterns of potential WNV vectors remain unanswered.

Host Feeding

Of the 23 mosquito species collected in CDC light traps, resting boxes, and a horse-baited stable trap, during my study, at least some individual females from 14 species had blood fed. Twelve species (fifty eight individuals) were positive for horse DNA: *Aedes vexans, Anopheles crucians, Anopheles quadrimaculatus, Culex nigripalpus, Culex salinarius, Culex erraticus, Mansonia titillans, Coquillettidia perturbans, Ochleratatus infirmatus, Ochleratatus mitchellae, Psorophora columbia,* and *Psorophor ciliata.* Individual females from eight mosquito species (*Ma. titillans, Cx. erraticus, Cx. salinarius, Cx.nigripalpus Ae. vexans, An. crucians, Oc. infirmatus,* and *Ps. columbiae*) blood fed on human (n = 48). *Mansonia titillans* blood fed primarily on mammals at my Alachua County, Florida study site. There was, however, a single *Ma. titillans* blood meal identification from a chicken. If *Ma. titillans* is a competent vector of WNV, then it could, under certain circumstances, serve as an epizootic or epidemic vector to

horses and humans in north Florida. Further work, such as vector competence studies and WNV screening of mosquito pools, can help clarify the potential role of this species in WNV transmission. There have been WNV isolations from *Ma. titillans*, but to my knowledge no work has been done to test vector competence. A major factor that may preclude *Ma. titillans* from vectoring WNV to humans and horses is that the seasonal abundance peaks late in the fall. *Mansonia titillans* is not active early in the spring during WNV amplification in the bird population. Instead, this species becomes active later in the season and is not likely to encounter a WNV positive bird prior to blood feeding on horses and humans.

Culex erraticus is another species that may be worth studying further because this species is an opportunistic feeder, was found at the site frequently, and is considered a competent vector of EEEV (Cupp et al. 2004). While competence for one type of arbovirus does not necessarily correlate with competence for another (Hardy et al. 1983), this species has had several isolations of WNV in the US. Vector competency studies of *Cx. erraticus* for WNV should be done. Vector competency studies indicate that other members of the genus *Culex (Cx. nigripalpus, Cx. quinquefasciatus,* and *Cx. tarsalis)* are moderate to excellent vectors of WNV (Turell et al. 2005). Laboratory studies show that *Cx. erraticus* is a long-lived species, which could contribute to its potential as a WNV vector (Kline et al. 1987).

The horse-baited stable trap used in this study attempted to collect a representation of mosquitoes entering the trap throughout the entire night. Many studies collect for short periods of time directly off the horse or bait animal. These collections record the species of biting flies that are attracted to the bait animal, but may not truly represent the species that are feeding on the animal. In this study the majority of the mosquitoes (518/528, 98%) that were collected from the horse-baited stable trap were blood fed. When the blood was analyzed by PCR it was

confirmed that, all the blood meals were derived from the horse (Chapter 5). Overall, the trap collected a small number of mosquitoes and future experiments could include modifications of the construction of the openings to allow for easier entrance of more mosquitoes and to make it more difficult for them to exit. In an experimental release of mosquitoes inside the stall, many were able to escape from the trap. Perhaps by adding an upward sloping baffle on the interior of the trap, fewer mosquitoes would have been able to exit the trap.

Surveillance

None of the sentinel animals (three horses, two chickens) tested positive for any of the arboviruses being monitored (SLEV, EEE, HJ, and WNV) in 2005 and 2006. Sentinel chickens are routinely utilized in arbovirus surveillance programs in the state of Florida and are considered reliable predictors of arboviral activity in an area (Day and Lewis 1991). The location for monitoring sentinel animals was chosen because horse cases occurred at the site in 2001. This site would probably be appropriate to continue monitoring for virus in future studies, perhaps the addition of more sentinel chickens to the flock would increase the possibility of arbovirus detection.

From the mosquito pools (n = 359) tested for arbovirus (WNV, SLEV and EEEV), there was one positive SLEV identification. To my knowledge this is the first time SLEV has been isolated from a pool of *Ma. titillans*. *Mansonia titillans* is involved with transmission of an alphavirus Venezuelan equine encephalomyelitis virus (family *Togaviridae,* genus *Alphavirus*, VEEV) (Mendez et al. 2001, Turell et al. 2000). Future studies determining vector competence of *Mansonia* mosquitoes for WNV and SLEV would be valuable to determine if this genus may play a secondary role in transmission of arboviruses in Florida.

Microenvironment and Weather

As part of this study, microenvironment and weather were correlated with mosquito abundance and diversity. Several differences were found for trap location at the study site, with the highest mosquito trap location being in Bivens Forest (Chapter 4). Weather conditions studied (rainfall, temperature and wind speed) were not significantly correlated with mosquito species abundance. This may have been due to the yearly meteorological differences at the site and may take a longer study period to establish how the climate affects the mosquito populations at this site. Previous field studies conducted in warm tropical climates have found an association between meteorological and landscape conditions and the incidence of mosquito borne disease (Reisen et al. 1993, Dhileepan 1996, Hu et al. 2006). In the United States, Mirimontes et al. (2006) found that high temperature and agricultural land use was associated with an increased incidence of WNV. In Georgia, urbanization was found to increase risk of human WNV infection (Gibbs et al. 2006). In Texas, mosquito vector populations were correlated with temperature, precipitation, and canopy cover (Bolling et al. 2005). In Rhode Island, precipitation was the factor most closely associated with arbovirus activity (Takeda et al. 2003), and in Florida, spring drought followed by rain was specifically associated with incidence of WNV (Shaman et al. 2005). Given the multiple correlations of WNV activity with climatological (long term weather) conditions, perhaps the collection of weather data at this site for a longer period of time would be informative.

Extrinsic Risk Factors of WNV to Horses

In the study of the extrinsic risk factors of WNV to horses, several factors were close to significant and warrant further study. The presence of water on the property should be investigated to determine the association of type of water present and risk of WNV infection in horses. Additionally, it would be interesting to study the relationship of fan use and mosquito

activity. Time of day the fans are used and the type of fan used could be compared experimentally with the abundance of mosquitoes entering a stable. Investigating these factors could not only help in evaluating WNV risk to horses, but could also be useful in further describing mosquito population dynamics in an agricultural setting. The strongest protective factor to horses for WNV was vaccination status. The negative comparison group in this study showed signs of arboviral infection and may not have represented a true negative control. A case control study would be a useful future study.

Conclusions

Mosquitoes commonly fed on horses, and several *Culex* spp were abundant and temporally correlated with arboviral activity in north central Florida. Future experiments that focus on arbovirus transmission to horses should consider *Cx. erraticus, Cx. nigripalpus,* and *Cx. salinarius* as possible epizootic vectors of EEEV and WNV to horses. The seasonal abundance of these three mosquitoes, and their vector competence in a laboratory setting combined with the blood meals from horse make them strong candidates as potential arboviral vectors to horses in Florida. The single most important preventative measure a horse owner can take is to vaccinate the horse against WNV twice a year. This is especially important in Florida where mosquito activity can occur year round.

APPENDIX A
ARBOVIRUS CASE INFORMATION FORM

CHARLES H. BRONSON
COMMISSIONER

Florida Department of Agriculture & Consumer Services
Division of Animal Industry
Bureau of Animal Disease Control

Arboviral Encephalitis
Case Information Form

585.145, Florida Statutes

Contact:

Dr. Michael A. Short
Equine Programs
Rm 329, 407 S. Calhoun St.
Tallahassee, FL 32399-0800
850/410-0901; Fax: 410-0919

www.doacs.state.fl.us/ai

Note: All documents and attachments submitted with this request are subject to public review pursuant to Chapter 119, F.S.

Submitter: **Please send this completed form along with collected samples to the Kissimmee Diagnostic Laboratory at: 2700 N John Young Pkwy, Kissimmee, FL 34741 Phone (321) 697-1400**

FOR LAB USE ONLY

If submitting split samples, send copies of completed form (both pages) to each laboratory used. If samples are not being submitted, please send the completed form to Dr. Michael A. Short, Division of Animal Industry, Fax 850-410-0919. Hard copies can be mailed to the address shown above.

County _____ Date Reported _____

Premises GPS (5 decimal digits)

Latitude _____ Longitude _____ Premises ID Number _____

FDACS/USDA Veterinarian(s) or Inspector(s) Assigned: _____

Reported By	Name	Title/Occupation
	Business/Affiliation	
	Mailing Address	Physical Address (if different)
	Phone #	Fax #
	Mobile #	Pager #
	Email	
Premises Information	Name	Title/Occupation
	Premises/Farm Name	
	Mailing Address	Physical Address (if different) (***Where Horse Resides***)
	Phone #	Fax #
	Mobile #	Pager #
	Email	

Arboviral Encephalitis
Case Information Form (continued)

Horse Information	Name/Animal Identification	Date of onset of clinical symptoms
	Breed	Age
	Sex (Male/Female/Gelding)	Vaccination Status (History)
	Status of Horse: ____ Alive ____ Dead ____ Critical Recovering as of (Date):	Date of Death: Buried?___ Yes ___ No
	Showing clinical symptoms?____ Yes ____ No	Method of Death: _____ Natural causes _____ Euthanasia _____ Other:

Samples	Number of samples taken.	Date samples taken:
	Samples submitted to FDACS Kissimmee Diagnostic Laboratory Sample type:____ Blood ____ Brain_____ Other: Date Sent:	
	Samples submitted to USDA National Veterinary Services Laboratory (NVSL) Sample type:____ Blood____ Brain____ Other: Date Sent:	
	Samples submitted to Florida DOH Laboratory Sample type: ____ Blood ____ Brain ____ Other: Date Sent:	

Clinical Presentation/History	History:
	Clinical Presentation: ___Apprehension Other: ___Depression ___Elevated Temperature ___Head Shaking ___Muscle Twitching ___Incoordination ___Weakness of Hind Limbs ___Inability to Stand ___Aimless Wandering ___Head Pressing ___Listlessness

Comments/Additional Information:
Attach additional pages as needed.

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APPENDIX B
ENCEPHALITIS SURVEY

1. What best describes the activity of the horse that became ill:

2. How many years has the horse that became ill been at this address:

3. How many horses were on the property when it became ill?

4. How many other horses become ill/displayed neurological symptoms in:
2000 _____ 2001 _____ 2002 _____ 2003 _____

5. How many other horses have been diagnosed with WNV or EEE:
2000 _____ 2001 _____ 2002 _____ 2003 _____

6. Did the horse survive its clinical symptoms?
   If its answer is no to question 6, skip to question 13.

7. Did the horse recover to complete activity after its clinical signs?
   If yes, how long did it take? (months)

8. A. There are changes in your horses personality?
   B. Does the horse act depressed?
   C. Are there gait abnormalities?
   D. Does the horse shake spontaneously?
   E. Does the horse shake after being ridden?
   F. Does the horse act weak or is incapable of maintaining its own weight?
   G. Is there a loss of muscle mass?
   H. If there is muscle loss is it in a specific area or general loss?

9. If the horse survived, do you still posses it?

10. If the horse was sold, did you receive the expected value?

11. If you did not receive the expected value, estimate your loss.

12. If the horse was sold, how much did the illness increase your investment?

13. If the horse did not survive, estimate its replacement value.

14. When the horse was ill, what were your veterinarian costs?

15. If you had to miss work, estimate your loss?

16. A. When the horse became ill was vaccinated against WNV?
    B. If the answer is yes, report when month/year.

C. Were the other horses on the property vaccinated against WNV?

D. How many times were the horses vaccinated in the 2001?
_____Once ____ Twice _____Three times ____Four times
E. How many times were the horses vaccinated in the 2002?
_____Once ____ Twice _____Three times _____Four times
F. How many times were the horses vaccinated in the 2003?
_____Once ______Twice _____Three times ____Four times
G. What months were they vaccinated?
H. Was the horse that became ill vaccinated against EEE?
I. If the answer is if, report when month/year.
J. Were the other horses on the property vaccinated against EEE?
K. How many times were the horses vaccinated in the 2001?
_____Once ____ Twice _____Three times ____Four times
L. How many times were the horses vaccinated in the 2002?
_____Once ____ Twice _____Three times _____Four times
M. How many times were the horses vaccinated in the 2003?
_____Once ______Twice _____Three times ____Four times
N. What months were they vaccinated?

17. Who applied the vaccine for WNV?
_____Myself _____Veterinarian _____Other
Who applied the vaccine for EEE?
_____Myself _____Veterinarian _____ Other

18. What was the cost to vaccinate on a per horse basis against WNV and EEE in 2000?_____ 2001?_____ 2002?_____ 2003?_____

19. What best describes the way in which the horse was treated when ill?
_____ Stabled all day and night. _____ Stabled and let out 2-4 hours during the day.
_____ Stabled in the afternoon/outside in the morning and night.
_____ Stabled all day/outside all night._____ Stabled at night/outside all day.
_____ Stabled and let out 2-4 hours in the evening _____ Outside 24 hours

20. When let out, the horse was in: _____ Pasture (0.5 acres) _____ Grass Paddock
_____ Sand Paddock

21. If stabled, how often were the stalls cleaned? _____ Every month _____ 2 times a month
_____ every week _____Daily

22. Are there fans in the stable?
If yes, how often are they run? _____All day ____Only when necessary _____ Never

23. What best describes the cover of the stable? _____boards with openings
_____Solid wood or cement _____Open shed

24. After rain, is there temporary standing water on the property?

25. Mark the different water sources for the horse. ____Bucket _____automatic
____natural
_____water tank

26. Describe the type of water that exists on the property. _____ River _____ Stream
_____ Lagoon or pool _____ Marsh or wetland
27. What best describes the canopy cover? _____Over barn ____Over grass
_____Over both

28. How many piles of debris exist near the stable, or the area of horse activity?
29. When in the stable, do you notice great, medium, or minimum activity of flies and/or mosquitoes? ____Severe _____Medium _____Minimum ____ None

30. What best describes the contents of the repellant used during the time in which the horse was ill? _____Permethrin _____Citronella _____Skin so soft _____ Organic
How frequently was the repellant used? ___ As needed ____ once daily ____ 2-3 times a week
___ Weekly _____ Monthly _____ None

31. How often did you use a flysheet? ___ Never ____ Occasionally ___ Continuously

32. During the time of your horse's illness, did you notice any dead birds on your property?
____ no ____ yes
During that same time, were there other ill animals? If yes, please list:
_____

Reproductive effects. Please it responds if there was breeding activity on the premises where its horse resided during the time of its disease.

33. Was there any breeding activity on the property since 2000? ____ No ____ Yes
2001? ____ No _____Yes 2002? ____ No _____Yes 2003? ____ No _____Yes

34. In 2000, how many mares checked pregnant at 30 days? _____
In 2001, how many of these mares foaled? _____
In 2001, how many mares checked pregnant at 30 days? _____

In 2002, how many of these mares foaled? _____
In 2002, how many mares checked pregnant at 30 days? _____
In 2003, how many of these mares foaled? _____
In 2003, how many mares checked pregnant at 30 days? _____
In 2004 how many of these mares foaled? _____

35. Did you vaccinate your mare against the WNV during pregnancy? ____ no _____ yes

36. Were there any abortions after the vaccine against WNV was administered during the gestation or at the end of the pregnancy? _____ no ____ yes
If the answer is yes, were other vaccines that were administered at the same time or shortly
before the abortion? _____ no _____ yes

37. Have there been abortions during the months of autumn independently of vaccines administered on the property or farm? _____ no _____ yes

APPENDIX C
SURVEY REQUEST LETTER

UNIVERSITY OF FLORIDA

February 18, 2004

To whom it may concern,

In an effort to gain critical information on the West Nile Virus and Eastern Equine Encephalitis outbreaks since 2001 in Florida, the University of Florida, in collaboration with the Florida Department of Agriculture and Consumer Services and the United States Department of Agriculture, are asking for your assistance in filling out the enclosed survey. This information will help us understand the natural course of mosquito transmitted encephalitis that threatens Florida horses yearly.

Your horse was reported to have exhibited clinical signs consistent with either West Nile virus or Eastern Equine Encephalitis virus. Although these signs may or may not have been confirmed by testing, the enclosed survey verifies vaccination information submitted at the time of testing, as well as, gathering further information regarding herd health and management. This will allow us to identify risk and management factors that we can then make recommendations about to the horse owning public.

Your participation in this study is of great importance, and your response is much appreciated. As a token of our appreciation, please use the enclosed gift certificate.

Enclosed is a postage-paid envelope so that you can return the survey to the University of Florida as soon as possible. You may also fax this information to Ashley Cunningham at 352-392-8289.

We hope that you will assist us in this important endeavor.

Respectfully,

The Veterinary Class of 2008
Maureen Long, DVM, PhD, DACVIM-LA
Assistant Professor
Large Animal Clinical Sciences
Enclosures (2)

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BIOGRAPHICAL SKETCH

Leslie Rios was born in Seattle Washington. She received her bachelor's degree from Western Washington University in 1998. She continued her education at Oregon State University studying entomology. She received her master's degree in 2000. She then worked for the University of Alabama at Birmingham. There she studied West Nile virus in mosquito and bird populations. It was there that she fell in love with the field of medical entomology and went on to receive her PhD at the University of Florida.