3-31-2014

Overwintering and Early Season Amplification of Eastern Equine Encephalitis Virus in the Southeastern United States

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Overwintering and Early Season Amplification of Eastern Equine Encephalitis Virus in the Southeastern United States

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Date of Approval:
March 31, 2014

Keywords: Arbovirus, Winter Transmission, Ecology, Reservoir Hosts

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DEDICATION

I would also like to thank my parents for their positive support and for always being there for when I needed reassurance. They helped me achieve everything that I currently have and have provided me with a foundation for everything to come. Without their encouragement, I would never have gotten this far. I would also like to thank everyone who has helped me get through the dissertation process emotionally, especially Collin, Kelley, Amanda, Jenn, and Amruta. Thank you guys so much for all the distractions, support, and for putting up with all of my craziness. You are the best friends anyone could ask for. I would also like to thank my major professor and committee for all of the help they have given me throughout this entire process.
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ABSTRACT

Eastern equine encephalitis virus (EEEV) is a highly pathogenic arbovirus that causes severe disease, with a mortality rate of approximately 30-35% in humans and 80-90% in horses. Studies dating back to the 1930’s have identified many of the epidemiological and ecological aspects of the virus. However, there are several aspects of EEEV’s transmission cycle that remain unclear. In the northeastern states, transmission is seasonal, peaking in the late summer months, while in Florida there is year-round transmission of EEEV. Recent phylogenetic studies have also suggested that Florida may serve as a reservoir for EEEV; the virus may periodically be introduced from Florida to the northeastern US where it locally amplifies, overwinters, and can remain stable for several years. How EEEV is able to migrate from Florida and how it persists during the winter in North America is not yet known, however several theories exist and are examined further by this research. The first part of this study investigates the hypothesis that snakes may serve as overwintering reservoir hosts for EEEV. Rates of exposure and infection of wild snakes were examined by testing serum samples from wild snakes at a focus of EEEV in Alabama. Two species of vipers, the cottonmouth and the copperhead, were found to be positive for EEEV RNA. The second part of the study attempts to identify the hosts and vectors of enzootic winter transmission of EEEV in Florida, with a focus on avian host preference. EEEV was detected in two mosquito species, *Culiseta melanura* and *Anopheles quadrimaculatus*, and were both from the month of February. In addition, the results also suggest that EEEV vectors preferentially feed upon wading birds during the winter months.
INTRODUCTION

Eastern equine encephalitis virus (EEEV) is one of the most pathogenic arboviruses, with a high mortality rate of approximately 30-35% in humans and 80-90% in horses.\textsuperscript{1} EEEV is a member of the genus \textit{Alphavirus} and family \textit{Togaviridae}, and possesses a positive sense RNA genome of approximately 11.7 kb.\textsuperscript{1} The disease was first described in horses in 1831 in Massachusetts when 75 horses died of encephalitic symptoms;\textsuperscript{2} however, the virus wasn’t isolated from horses until 1933\textsuperscript{3} and the first human case wasn’t identified until 1938.\textsuperscript{4}

The distribution of EEEV ranges throughout North, Central, and South America as well as the Caribbean.\textsuperscript{5} Although the virus is found throughout the Americas, both hemagglutination inhibition assays and phylogenetic studies have indicated that the North and South American isolates are separate antigenic variants.\textsuperscript{6,7} Isolates from North America have been shown to consist of one lineage (I), while South American isolates belong to one of three lineages (II, III, and IV).\textsuperscript{7,8} The North American variant is also considered to be more pathogenic, and differs from the South American variant in terms of ecology, and mosquito and vertebrate hosts.\textsuperscript{9} In the United States, cases occur more frequently in the Atlantic and Gulf Coast states, with approximately six human cases and 200 horse cases per year. The first documented case in Florida occurred in 1952.\textsuperscript{10} Since then, Florida has had more human and horse cases than any other state, with an average of 1 to 2 human and 70 horse cases per year (FDOH unpublished data). Other states with increased human cases include Massachusetts, Georgia, and New Jersey, although each of these states has less than half the cases of Florida. Florida is also unique in that
EEEV transmission occurs year-round. In the northeastern states, transmission is seasonal, peaking in the late summer months. EEEV can cause either a systemic or encephalitic infection. Symptoms of the systemic illness include chills, fever, malaise, arthralgia, and myalgia. Symptoms usually resolve when there is no central nervous system (CNS) involvement. When there is CNS involvement, the same symptoms manifest as well as the encephalitic ones: headache, restlessness, drowsiness, anorexia, vomiting, diarrhea, cyanosis, convulsions, and coma. In infants, the onset of encephalitis is abrupt, while in children and adults it manifests after several days of systemic infection. Death usually occurs within 2-10 days for 30-50% of infected individuals. Approximately two thirds of the individuals who recover from infection suffer from chronic neurological sequelae and many of those who survive the initial infection may die within a few years. The highest rates of mortality occur in children and the elderly, with age playing a role in the development of more severe sequelae.

A similar sequence of infection has also been seen in animals. Mouse models have indicated that there is a self-limiting replication phase in peripheral tissue followed by a fatal CNS phase. This spread to the CNS has been shown to occur as early as 24-48 hours after infection. However, the incubation period in horses and humans is often considered to be 3-10 days. After initial replication in the skeletal muscle of the bite site, the virus can spread to other skeletal muscles and lymphatic tissues. Mouse models have indicated that osteoblasts are readily infected and are involved with early virus amplification. The replication within osteoblasts is especially important since they are specialized fibroblasts responsible for bone formation and infection of these cells results in a higher titer of viremia. These cells are more prominent in the young and their numbers decrease with age, which may explain why there is a
higher incidence of neuroinvasion, encephalitis, and death in the young and why older animals may be refractory to encephalitis after being infected. The target cells for EEEV replication are those of the nervous system, mainly neurons, and damage to these cells is severe and potentially irreversible. EEEV has been shown to be directly cytopathic for neurons. The outcome of infection is determined by whether the neurons survive infection and survival is determined by the virulence of the virus and the age of the host at the time of infection. This is because immature, developing neurons are more supportive of viral replication and mature neurons may resist virus induced apoptosis (cell death) by expressing cellular inhibitors of apoptosis. The mechanism by which EEEV enters the CNS is not entirely clear; however, once the virus is able to enter the CNS, it can spread from cell to cell or through the cerebral spinal fluid.

Although there are few human cases ever year, EEEV has a strong social and economical impact. Mild infections with EEEV have been shown to cost over $21,000 per case, while children who suffer from severe sequelae can incur lifetime costs upwards of $3 million. The economic impact of these epidemics to the equine industry are also severe, with costs associated with treatment of EEEV totaling over $1 million per year. There is no effective treatment for EEEV infections and all therapies are considered to be supportive. There is a vaccine for horses; however, annual boosters are needed in order to maintain neutralizing antibodies and horses residing in highly endemic areas require multiple boosters every year. These vaccines are usually available as multivalent vaccines, in combination with other viruses affecting horses or tetanus toxoid.

EEEV has been shown to infect horses, humans, birds and other animals and is maintained in an enzootic cycle between ornithophilic mosquitoes and avian reservoir hosts. Passerine birds are considered to be the major reservoir hosts for EEEV in North America, with
enzootic transmission between avian hosts mediated by ornithophilic *Culiseta melanura* mosquitoes, particularly in freshwater swamp foci.\(^1,26,27\) The virus was first isolated from birds and from *Culiseta melanura* mosquitoes in 1951.\(^28,29\) Other mosquito species including *Aedes vexans*, *Aedes sollicitans*, and *Coquillettidia perturbans*, which feed upon mammals and birds, have been implicated in transmitting EEEV from birds to horses and humans.\(^30,31,32\) Shifts in feeding patterns of bridge vectors from feeding on avian to mammalian hosts during the transmission season may help to facilitate transmission to horses and humans.\(^33,34,35\) Mosquito feeding preference has been shown to vary temporally,\(^36\) and may be due to mosquito abundance,\(^37\) host availability or behavior, host reproductive phenology,\(^34\) and winter severity.\(^35\) Many of the mosquitoes that can transmit EEEV also have crepuscular or nocturnal activity,\(^38,39\) and their feeding preferences may reflect the available hosts during this time period.

The ability of a mosquito to serve as a disease vector depends on a variety of factors. One of the important factors is that the mosquito species known or suspected to transmit EEEV are feeding on potentially infected hosts. The catholic feeding preference of many of the bridge vectors allows for many animals to potentially be exposed to the virus. However, many of these animals may be dead end hosts and not have enough circulating viremia to infect mosquitoes. Several serosurveys have been conducted in order to identify which species are frequently exposed to EEEV in nature. EEEV has been shown to infect horses, humans, birds and other animals.\(^25\) Many species of birds have been identified as exposed to EEEV in nature, and more than 50 species of birds have been naturally or experimentally infected with EEEV.\(^25,40,41\) Non-native birds have also been shown to be quite vulnerable to EEEV.\(^42,43,44\) Conversely, many mammals have also been exposed to EEEV in the past;\(^45,46,47,48,49,50\) however, few isolates of virus have come from these mammals and many are considered to be dead end hosts. In
particular, white tailed deer have been exposed to EEEV in the wild\textsuperscript{45,51} and several cases of clinical disease have also been reported.\textsuperscript{47,52} Interestingly, small mammals are considered to be the reservoir hosts for the South American variant of eastern equine encephalitis virus with \textit{Culex} (\textit{Melanoconion}) species serving as the primary vectors.\textsuperscript{1,53} The majority of human serosurveys have focused on the South American variant of EEEV, with seroprevalence ranging from 3-66\%.\textsuperscript{54,55,56} Studies in enzootic locations in the northeast found a seroprevalence rate around 2\%.\textsuperscript{57,58} In addition, antibodies to EEEV have also been found in several species of reptiles and amphibians.\textsuperscript{59,60,61}

Another important factor in the transmission of EEEV is how efficient the mosquito is at spreading the virus. Vector competency refers to the genetic factors of the mosquito that affect how competent the mosquito is as a vector.\textsuperscript{62,63} Upon feeding on an infected host, the virus travels to the mosquito’s midgut, replicates and exits to the hemocoel, where the virus can then disseminate to infect secondary organs.\textsuperscript{62} The virus will again replicate in the salivary glands and can be transmitted to another host during the next blood meal. Each step represents a potential barrier to infection within the mosquito. Vectorial capacity takes vector competency into account as well as the behavioral and environmental characteristics of the mosquito in determining its importance as a vector.\textsuperscript{62,64} One important intrinsic factor is how long it takes the pathogen to disseminate to the salivary glands, known as the extrinsic incubation period.\textsuperscript{65} \textit{Cs. melanura} is considered to be highly competent for EEEV, with virus disseminating to the salivary glands in as little as 2-3 days.\textsuperscript{66,67} Other species of mosquitoes require a 1-2 week incubation period before being able to transmit the virus.\textsuperscript{68,69,70} There are several species of mosquitoes that are known or suspected bridge vectors of EEEV.\textsuperscript{68}
Most studies of EEEV have concentrated upon foci in the northern parts of the US. In the northeastern states, transmission is seasonal, peaking in the late summer months.\(^1\) *Cs. melanura* is also highly prevalent in enzootic sites in the northeast.\(^71, 72, 73\) This differs from the southeast, where several studies have found virus circulating during patterns of high *Culex erraticus* and low *Cs. melanura* populations.\(^74\) In addition, it has been hypothesized that locations that have undergone drastic habitat changes, such as deforestation, lead to the gradual replacement of *Cs. melanura* by *Cx. erraticus*.\(^75\) Many of the bridge vectors may also differ between the two regions. In addition, domestic chickens are used in the southeast as avian sentinels but were not found to be useful for detecting EEEV activity in the northeast.\(^76\) Chickens are chosen as sentinels because they are susceptible to infection and are capable of developing detectable levels of antibodies, the infection is not life-threatening, and significant viremia does not develop. Thus they are non-infectious to handlers, mosquitoes, and other chickens.\(^77, 78\) In Florida, the chicken sentinel program has been in place since 1978,\(^79\) however, only 14 counties have maintained sentinel flocks from 1978 to the present and active surveillance is non-existent in most Florida counties.\(^80\) In addition, not all of the counties that are involved with the chicken sentinel program leave their chickens out year-round, so sentinel transmission rates may be under exaggerated.

Recent phylogenetic studies have also suggested that Florida may serve as a reservoir for EEEV; the virus may periodically be introduced from Florida to the northeastern US, where it locally amplifies, overwinters, and can remain stable for several years.\(^81, 82, 83\) It is possible that a combination of local overwintering and re-introductions from southern foci allow the virus to initiate the transmission cycle every year in the northeast. In many locations, early spring transmission of virus is seen among birds before the peak transmission season occurs.\(^27, 84\) How
the virus is able to overwinter and re-enter the avian population remains undetermined. One possibility is that the virus is re-introduced by migrating birds from locations such as Latin America or South America. This is unlikely due to the antigenic and pathogenic differences found between the North American and South American variants.\textsuperscript{85} In addition, few virus isolates from migrating birds from these locations have been found.\textsuperscript{86} A second possibility is the recrudescence hypothesis, where the virus is able to reside in birds during the winter months in a latent form and transforms each spring into a replicating form in response to stressors such as migration or hormonal changes in the bird.\textsuperscript{26, 27} This was recently tested in gray catbirds and also shown not to be the case.\textsuperscript{87} Another hypothesis that has been examined is whether or not the virus is overwintering in the mosquito. Studies conducted in the southeastern US, however, have been unable to identify any virus in collections of overwintering adult mosquitoes.\textsuperscript{88} Those species in the northeast that overwinter as adults have also been tested for virus and found to be negative.\textsuperscript{89} In the northeast, many of the vector species of EEEV overwinter in the egg or larval stage.\textsuperscript{90} \textit{C. melanura} overwinters in the larval stage in the northeast; however, there is no evidence of vertical transmission of EEEV in this species or others.\textsuperscript{91, 92, 93, 94}

There remain two other alternative hypotheses that should be investigated further. One is that perhaps there is another reservoir host involved with the overwintering and early season amplification of EEEV. Studies in the 1950’s and 1960’s focused on the potential of ectotherms to serve as potential overwintering hosts for the virus, a hypothesis that has recently been re-investigated. Studies conducted in Alabama indicated that several mosquito species known to feed on ectothermic hosts had pools that were positive for EEEV viral RNA.\textsuperscript{74, 95, 96} The majority of \textit{Culex peccator} and \textit{Uranotaenia sapphirina} blood meals came from snakes and frogs. Snakes have also been shown to be exposed to EEEV in the wild.\textsuperscript{48, 59, 60, 61} Past studies have indicated
that several snake species are susceptible to infection with long lasting, high titers of viremia that is temperature dependent. One study found that viremia lasted from 3-105 days depending on temperature and that post-hibernation, snakes were still viremic for a few months. Most of the research looking at arboviruses and reptiles has focused on another alphavirus, western equine encephalitis virus (WEEV). WEEV is genetically related to EEEV and is thought to have been the result of a recombination event between EEEV-like and Sindbis-like viruses. Previous studies have shown garter snakes to be competent hosts for WEEV, with long lasting levels of high viremia. These studies demonstrated the ability of Culex tarsalis to transmit WEEV to naïve snakes as well as the vertical transmission of the virus from infected mothers to offspring. WEEV viremia has been shown to last 70 days post hibernation in garter snakes when infected before hibernation. WEEV has also been isolated from snakes in the wild during a time of no mosquito activity.

Another possibility for the re-introduction of virus may be from migrating birds taking the virus back with them after overwintering in the southeast. Most of the studies examining reservoir competence have focused on passerine birds. Studies in the northeast have indicated that permanent resident and summer resident birds have antibodies to EEEV more often than transient and winter resident birds and there is some evidence toward migrating birds playing a role in transportation of EEEV. This makes sense due to the majority of cases occurring during the summer months. Many of these studies conclude that since such a high percentage of permanent residents have antibodies, that migratory birds play a minimal role in the introduction of EEEV. However, these studies focused on passerine birds and many don’t take into account the location of the wintering grounds for these bird species.
Recent studies have indicated that wading birds (Ciconiiformes) are preferred hosts of potential bridge vectors of EEEV in the southeast.\textsuperscript{36, 108, 109, 110} Few studies have looked at the exposure of wading birds to EEEV in comparison to the numbers of studies that have focused on passerine birds; however, one seroprevalence study conducted in Louisiana found that over 80% of yellow-crowned night herons were seropositive for EEEV,\textsuperscript{106} higher than any of the Passeriform birds, and virus was isolated from one of the nestlings.\textsuperscript{111} Additional studies have implicated a wide variety of wading bird species as potential enzootic hosts for EEEV as well.\textsuperscript{40, 45, 112, 113, 114} In 1962, Herman reported 8 species of Ciconiiformes (common egret, snowy egret, black-crowned night heron, green heron, little blue heron, Louisiana heron, yellow-crowned night heron, and white ibis) as either being naturally or experimentally infected with EEEV.\textsuperscript{40} Experimentally infected wading and water birds have been shown to have high enough viremia to infect mosquitoes.\textsuperscript{41, 115, 116}

This research attempts to further describe potential pathways for the overwintering and early season amplification of EEEV and build upon previous research in this field. There are several parts of the EEEV transmission cycle that are missing or not completely described. Two hypotheses, local overwintering in snakes and re-introductions of virus by migrating birds, are examined herein. The first part of this study attempts to identify circulating viremia in snake serum collected from wild snakes in Alabama. It is important to know if snakes have active viral infections, in addition to exposure to the virus, if they are going to serve as potential overwintering hosts for the virus. The second part of this study attempts to identify the hosts and vectors of enzootic winter transmission of EEEV in Florida, with a focus on avian host preference. If the virus is migrating from Florida to other locations after the winter months, it is important to know what species are being fed upon during this time period.
References


CHAPTER ONE

Detection of Eastern Equine Encephalomyelitis Virus RNA in North American Snakes

Note to Reader

This chapter has been previously published (Bingham, AB, et al., 2012. Detection of Eastern Equine Encephalomyelitis Virus RNA in North American Snakes. Am J Trop Med Hyg 87(6): 1140-1144.) and is utilized with permission from the publisher.

Abstract

The role of non-avian vertebrates in the ecology of eastern equine encephalomyelitis virus (EEEV) is unresolved, but mounting evidence supports a potential role for snakes in the EEEV transmission cycle, especially as overwintering hosts. To determine rates of exposure and infection, we examined serum samples from wild snakes at a focus of EEEV in Alabama for viral RNA using quantitative reverse transcription polymerase chain reaction. Two species of vipers, the copperhead (Agkistrodon contortrix) and the cottonmouth (Agkistrodon piscivorus), were found to be positive for EEEV RNA using this assay. Prevalence of EEEV RNA was higher in seropositive snakes than seronegative snakes. Positivity for the quantitative reverse transcription polymerase chain reaction in cottonmouths peaked in April and September. Body size and sex ratios were not significantly different between infected and uninfected snakes. These results support the hypothesis that snakes are involved in the ecology of EEEV in North America, possibly as overwintering hosts for the virus.
Introduction

Eastern equine encephalomyelitis virus (EEEV; family Togaviridae, genus Alphavirus) is an extremely pathogenic arbovirus endemic to New England south to Florida, extending west as far as Michigan. This virus circulates year-round in Florida, but outside of Florida its transmission is seasonal. Recent studies have suggested that the virus is periodically introduced from Florida to the northeastern United States, where it establishes itself in defined foci and is capable of maintaining itself for several seasons. How EEEV overwinters in these foci remains unresolved. However, recent studies have implicated ectothermic animals, snakes in particular, as potential overwintering reservoir hosts for EEEV. For example, studies on the ecology of EEEV conducted in the Tuskegee National Forest in Alabama documented the presence of EEEV in pools of Culex peccator, Culex territans, and Uranotaenia sapphirina mosquitoes, with some of the EEEV positive pools in these species detected early in the transmission season. These mosquito species feed primarily upon ectothermic hosts, with Cx. territans primarily feeding upon amphibians and Cx. peccator and Ur. sapphirina primarily feeding upon reptiles.

Laboratory studies have also supported the hypothesis that ectotherms might play a role in the overwintering of EEEV. Recently, it was reported that snakes experimentally infected with EEEV developed circulating levels of viremia that were sufficient to infect mosquitoes and maintained these potentially infectious viral titers for 7–10 days. This period was longer than the period that infectious titers persist in passerine birds, the accepted enzootic hosts for EEEV. Furthermore, viremic snakes, when induced to go into a hibernation-like state known as brumation, maintained a circulating viremia upon exiting brumation.
Snakes also appear to be commonly exposed to EEEV. A recent serosurvey of ectothermic species from a focus of EEEV transmission in Tuskegee National Forest in Alabama showed that more than 35% of the cottonmouths (*Agkistrodon piscivorus*), the ectothermic species most frequently fed upon by mosquitoes in Tuskegee National Forest, contained antibodies to EEEV. However, these data must be interpreted with caution because the presence of antibodies recognizing EEEV in these animals might merely reflect exposure to the virus, but not the development of a patent infection.

Snakes and other ectothermic animals mount relatively inefficient antibody responses to pathogens, suggesting that antibodies produced by these animals against EEEV might not be efficient in clearing the infection with the virus. In support of this hypothesis, plasma from cottonmouths experimentally infected with EEEV, while containing antibodies to EEEV that were detectable by the luminex assay, were found to lack detectible antiviral activity in plaque reduction neutralization assays. These findings suggested the hypothesis that snakes exposed to EEEV in the wild might maintain a low level of circulating virus. To test this hypothesis, plasma samples collected from snakes at Tuskegee National Forest were tested for the presence of EEEV by using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

**Materials and Methods**

Collection of plasma from snakes at the Tuskegee National Forest has been described in detail in a previous publication. In brief, samples were collected from an EEEV-endemic area in Tuskegee National Forest, located in east-central Alabama. Samples were collected during April–September 2007–2009 during surveys of the herpetofauna present at the site, as described. Procedures used for collection of blood samples were approved by the Institutional Review Board for Animal Use and Care of Auburn University. Blood samples (1 mL) were
collected from the caudal sinus with a 26-gauge heparinized syringe. At the time of blood draw, body size and sex of each snake were recorded. Animals were marked to prevent re-sampling and released at the point of capture. Blood samples were transferred to 1.5-mL microcentrifuge tubes, placed on wet ice, and transported to the laboratory. Samples were centrifuged briefly and the plasma was decanted from the cell pellet. The luminex assay (recognizing antibodies to EEEV) was then used to determine snake exposure to EEEV only, as described. Samples determined to be antibody positive by luminex were then tested by qRT-PCR for EEEV RNA. In addition, samples from 66 randomly selected seronegative snakes (11 per month, April-September) were tested by qRT-PCR for EEEV RNA.

Total RNA was prepared from 140 µL of plasma by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s protocol. The process was automated with the Qiacube system (QIAGEN), and isolated RNA (60 µL) was stored at −80°C. Each batch of 12 samples processed in the Qiacube consisted of 11 serum samples and one sham extraction as a negative control.

The qRT-PCR was performed using the iScript one step RT-PCR kit for probes (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. Primers and reaction conditions used to detect EEEV RNA were those recommended by Lambert and others, with the exception that reactions were performed in a final volume of 25 µL and used 5 µL of the RNA template. These primers produced an amplicon spanning positions 9298–9456 in the EEEV genome sequence (GenBank accession no. X67111). Samples (and associated sham extractions) were run in a 96-well plate format, with each plate containing two qRT-PCR negative control wells. Amplicons were detected by using a 5’ 6-FAM, 3’ BHQ1a-Q probe spanning positions 9411–9431 in the
EEEV genome. Samples producing a signal at a cycle threshold (C_t) value \( \leq 37 \) were considered putatively positive.

RNA samples found to be putatively positive in the screening assay were subjected to a confirmatory qRT-PCR assay provided by the Centers for Disease Control and Prevention (Atlanta, GA) to state Department of Health Laboratories conducting arboviral surveillance activities.\(^{20}\) The confirmatory assay used two primers (5’-ACCTTGCTGACGACCAGGTC-3’ and 5’-GTTGTTGGTGCCTCAATCCA-3’), which produced an amplicon spanning positions 9428–9497 in the EEEV genome. The sequence of the probe used in this assay was 5’-CTTGGAAGTGATGCAAATCCAACTCGACA-3’, which spanned positions 9449–9477 in the genome. The probe contained the same fluorescent and quencher molecules as the first qRT-PCR. Putatively positive samples that produced a detectable amplicon in the confirmatory assay (C_t < 40) were considered confirmed as positive for EEEV RNA. The biochemical limit of detection of both assays was determined to be \( \leq 1 \) plaque-forming unit when assayed against cultured viral stocks of known titers.

Virus isolation was attempted from all confirmed qRT-PCR–positive samples by inoculating individual T-25 flasks of confluent Vero cell cultures with 1 mL of plasma. Flasks were incubated for two hours at 37°C, with gentle rocking every 15 minutes. After the incubation, 9 mL of maintenance media (1 x Earle’s minimal essential medium, 2% fetal bovine serum, 200 U/mL penicillin, 200 µg/mL streptomycin, and 2.5 µg/mL amphotericin B) were added to each flask. Cells were then monitored daily for a cytopathic effect.

Fisher’s exact test was used to test for the significance of the proportion of qRT-PCR–positive samples in seropositive and seronegative snakes. The Pearson’s chi-square test was used to test differences in sex ratio in EEEV qRT-PCR–positive and EEEV qRT-PCR–negative
snakes. Statistical differences in body sizes of infected and uninfected snakes (separated by sex) were determined using a t-test. All analyses were performed by using SAS version 9.1 statistical software (SAS Institute, Cary, NC).

**Results**

Cottonmouths (*Agkistrodon piscivorus*) were the most common and commonly sampled snakes at our site, representing 41% of the ectotherm biomass, and were previously shown to exhibit high rates of EEEV exposure, with 35.4% of cottonmouth serum samples tested containing antibodies to EEEV. For this reason, initial studies concentrated upon determining if EEEV RNA could be detected in seropositive and seronegative cottonmouths. Although EEEV RNA was detected in seropositive and seronegative snakes, cottonmouths with detectable antibodies against EEEV in their serum were significantly more likely to be qRT-PCR positive than seronegative cottonmouths (P < 0.001, by Fisher’s exact test). Of the 66 seronegative cottonmouths tested, only one (1.5%) was qRT-PCR positive, while 12 (22.2%) of the 54 seropositive snakes were positive for EEEV RNA. The Ct values for the snakes ranged from 33.4 to 37 for the screening assay and from 30.9 to 40 for the confirmatory assay. Attempts to culture EEEV from all the qRT-PCR–positive samples were not successful.

Previous studies had shown that temporal distribution of EEEV exposure in cottonmouths (as measured by antibody positivity) was relatively constant throughout the transmission season, with some suggestion of an increased prevalence of seropositivity in the spring and fall. A similar biphasic distribution of qRT-PCR positivity in seropositive cottonmouths was seen (Figure 1.1). In April, 22.2% of the total cottonmouths tested (seropositive and seronegative) were qRT-PCR positive. The proportion of qRT-PCR positive cottonmouths then decreased through the May–July period, reaching a nadir of 3.2% in July, and began to increase again in
August. The single seropositive cottonmouth sample collected in September was found to be qRT-PCR-positive for EEEV (Figure 1.1).

Seropositive serum samples collected from other species of snakes exposed to EEEV at Tuskegee National Forest were then tested for EEEV RNA by qRT-PCR. Two of the eight snake species tested (the cottonmouth and the copperhead \([Agkistrodon contortrix]\)) were found to contain qRT-PCR positive animals. Of the three copperheads sampled, one was positive for EEEV RNA. Serum samples from two other seropositive snake species (Plain-bellied watersnake \([Nerodia erythrogaster]\) and black racer \([Coluber constrictor]\)) were not positive for EEEV RNA (Table 1.1).

Because host body size\(^8\) and behavior\(^21\) can have strong effects on selection of ectothermic hosts by mosquitoes, and thus vector-host contact rates (a major driver in pathogen exposure), we investigated potential relationships between EEEV prevalence and snake body size and sex. No difference was observed between mean body size (measured by snout-vent length) of male or female cottonmouths exposed to EEEV and those not exposed to EEEV (Figure 1.2, Panel A) (females: \(P = 0.5779, t = 0.5595, \text{df} = 61\); males: \(P = 0.2898, t = 1.0718, \text{df} = 43\)). A slightly greater body size (mean snout-vent length) was observed for cottonmouths that were qRT-PCR positive than those that were qRT-PCR negative (Figure 1.2, Panel B), although the difference was not statistically significant (females: \(P = 0.6962, t = 0.3923, \text{df} = 61\); males: \(P = 0.8798, t = 0.1521, \text{df} = 43\)). Female cottonmouths constituted a greater proportion of the EEEV qRT-PCR–positive snakes than males (Figure 1.3), although this ratio was not significantly different from the sex ratio of qRT-PCR–negative snakes \((\chi^2 = 0.142, \text{df} = 1, P = 0.707)\).
Discussion

The data presented demonstrate that snakes at Tuskegee National Forest were not only exposed to EEEV (antibody positive), but that a proportion of snakes have detectable infections (qRT-PCR positive for EEEV RNA). Two snake species (cottonmouth and copperhead), both of the genus *Agkistrodon*, were found to have detectable levels of EEEV in serum samples collected at our study site in Tuskegee National Forest. Cottonmouths, the greatest source of reptilian biomass at our site,\(^7\) were frequently exposed to and infected with EEEV. Interestingly, cottonmouth snakes are commonly targeted hosts by suspected bridge vectors of EEEV at this site as well.\(^7\),\(^8\)

To our knowledge, this is the first report of the detection of virus (as opposed to antibodies) detected in field-collected serum samples from ectothermic vertebrates. Karstad\(^22\) detected neutralizing antibodies to EEEV from wild ectotherms, but could not isolate virus from the samples. Dalrymple\(^23\) and others\(^11\) also detected EEEV antibodies in several ectothermic species, but made no mention of whether virus isolation was attempted for serum samples from these same hosts. However, other snakes have been shown to be competent hosts for western equine encephalomyelitis virus,\(^24,25,26,27\) an Alphavirus related to EEEV, with one study showing viremia in western equine encephalomyelitis virus–infected snakes lasting 70 days post brumation.\(^25\) These studies demonstrated the ability of *Culex tarsalis* to transmit WEEV to naïve snakes\(^28\) as well as the vertical transmission of the virus from infected mothers to offspring.\(^26\) WEEV has also been isolated from snakes in the wild during a time of no mosquito activity.\(^26\) Attempts to culture EEEV were unsuccessful in this study, which might reflect sample degradation because the serum samples had been subjected to multiple freeze–thaw cycles. Another possibility is that the relatively inefficient adaptive immune response of snakes\(^16\) was
insufficient to completely clear the infection, permitting the maintenance of a low-titer circulating viremia.

The data suggest that the proportion of qRT-PCR–positive cottonmouths was highest in the spring. These data are in concordance with those of previous laboratory studies, which demonstrated that garter snakes (Thamnophis sirtalis) experimentally infected with EEEV held at low temperatures (18°C) were found to maintain circulating viremias for longer periods than did animals held at higher temperatures (25°C or 30°C). It is possible this might be a consequence of the temperature dependence of the ectothermic adaptive immune system. During cooler months, snakes may not be able to raise body temperatures to levels that would enable them to clear infections because the adaptive immune response of ectotherms is more efficient at higher temperatures.16, 17, 29, 30, 31

The relationship between host body size and infection is complicated, and positive and negative associations have been found in different host/vector-borne pathogen systems. Body size can be affected by infection, when physiological cost of infection is high. Larger body size can contribute to greater infection through increased exposure because vectors are believed to feed more frequently upon larger hosts. In our own study, we found no significant difference in the body size of exposed and unexposed snakes. However, body size was slightly (but not significantly) larger in qRT-PCR–positive snakes than in qRT-PCR–negative snakes.

For many vertebrate pathogens, males have higher prevalence and intensity of infection because of their larger home ranges, mate attracting/guarding activities, and hormonal influences. In contrast, in this study, we found that compared with the uninfected population, females made up a larger proportion of the population of infected cottonmouths, although the difference was not statistically significant from that of the uninfected population.
The behavior of cottonmouth snakes may also make them a potential reservoir host for EEEV. In warmer climates, cottonmouths are active from March to October. There is no defined breeding season; breeding can occur year-round. In some locations the breeding season occurs in April-May and young cottonmouths are born in August-September. Another study conducted at TNF noted that the breeding season was August-September. The cottonmouth snake has been shown to tolerate lower temperatures better than many other snake species and is usually the last to go into brumation. In more southern locations, cottonmouths may not brumate at all and have been seen basking during the mornings of cooler months. The metabolic costs of this non-feeding period have also been shown to increase with increasing latitude. Some species of female mosquitoes (including Culex species) also overwinter in underground burrows and root holes which are sites that are also used as hibernacula by cottonmouths and other snakes. Cottonmouth snakes are usually found basking during the day, but are more active at night. Cottonmouths do most of their hunting at night, corresponding with the nocturnal activity of some of its food supply (some species of frogs and reptiles). They remain motionless for long periods of time in order to ambush prey, which also encourages mosquito feeding. The mosquito species that are known or suspected vectors of EEEV also have crepuscular or nocturnal activity and therefore have a greater chance of feeding on a cottonmouth snake than more diurnal snakes.

Previous laboratory studies demonstrated that snakes experimentally infected with EEEV can remain viremic through brumation, and that viremia in these animals is affected by the ambient temperature, with infected animals held at lower temperatures having lower viral titers of circulating virus, but maintaining viremia for longer periods than animals held at higher temperatures. One study found that viremia lasted from 3-105 days depending on
temperature and that post-brumation, snakes were still viremic for a few months.\textsuperscript{11} The demonstration that wild-caught snakes contain EEEV virus in circulating blood and that the proportion of animals with circulating viremia is highest in the spring months provides further support to the hypothesis that snakes play an important role in overwintering and early season enzootic amplification of EEEV.
### Tables and Figures

**Table 1.1** Proportion of Luminex-positive snakes that were positive by qRT-PCR for eastern equine encephalitis virus from Tuskegee National Forest, Alabama, USA

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
<th>No. tested</th>
<th>% qRT-PCR positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coluber constrictor</em></td>
<td>Black racer</td>
<td>4</td>
<td>0</td>
<td>ND*</td>
</tr>
<tr>
<td><em>Agkistrodon contortrix</em></td>
<td>Copperhead</td>
<td>3</td>
<td>33.3</td>
<td>0-87</td>
</tr>
<tr>
<td><em>Agkistrodon piscivorus</em></td>
<td>Cottonmouth</td>
<td>54</td>
<td>22.2</td>
<td>13-35</td>
</tr>
<tr>
<td><em>Storeria dekayi</em></td>
<td>Dekay’s Brownsnake</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td><em>Nerodia sipedon pleuralis</em></td>
<td>Midland Watersnake</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td><em>Nerodia erythrogaster</em></td>
<td>Plain-bellied Watersnake</td>
<td>7</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td><em>Diadophis punctatus</em></td>
<td>Ringneck Snake</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td><em>Crotalus horridus</em></td>
<td>Timber Rattlesnake</td>
<td>1</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND = not determined

qRT-PCR = quantitative reverse transcription polymerase chain reaction; CI = confidence interval, ND = not determined
Figure 1.1 Exposure to (Antibody +) and infection with (PCR +) eastern equine encephalomyelitis virus in cottonmouth snakes (*Agkistrodon piscivorus*) from Tuskegee National Forest, Alabama, USA. Data on seropositivity rates were taken from previously published sources.
Figure 1.2 Body size (snout-vent length) of male and female cottonmouth snakes (*Agkistrodon piscivorus*) exposed to (A) and infected with (B) eastern equine encephalomyelitis virus from Tuskegee National Forest, Alabama, USA. Error bars show standard deviation.
Figure 1.3 Sex ratio of eastern equine encephalomyelitis virus (EEEV) quantitative reverse transcription polymerase chain reaction (qRT-PCR)-positive (A) and EEEV qRT-PCR-negative (B) cottonmouth snakes (*Agkistrodon piscivorus*) from Tuskegee National Forest, Alabama, USA.
References


CHAPTER TWO

Field Investigations of Winter Transmission of Eastern Equine Encephalitis virus in Florida

Abstract

Field studies investigating the dynamics of winter transmission of eastern equine encephalitis virus (EEEV) were conducted in Tampa, Florida to identify EEEV vectors and their associated hosts. EEEV was detected in pools of *Culiseta melanura* and *Anopheles quadrimaculatus* in February of 2012 and 2013. During winter months, herons were major hosts for *Cs. melanura* and *Culex erraticus*, primary and epizootic vectors of EEEV, respectively. In summer months, *Cs. melanura* females were not encountered at the same sampling locations, while *Cx. erraticus* was still abundant, but took fewer blood meals from herons than in winter. Four bird species (black-crowned night heron, yellow-crowned night heron, anhinga, and great blue heron) were fed upon to a greater extent than their relative abundance predicted. Results suggest that EEEV vectors preferentially feed upon wading birds during the winter months suggesting that these birds may participate in maintaining EEEV during the winter in Florida.

Introduction

Eastern equine encephalitis virus (EEEV) is a highly pathogenic arbovirus that causes severe disease, with a mortality rate of approximately 30-35% in humans and 80-90% in horses.\(^1\) Approximately two thirds of the individuals that recover from the infection suffer from chronic neurological sequelae\(^1,2\) that can incur lifetime costs upwards of $3 million.\(^3\) In the US, cases occur most frequently in the Atlantic and Gulf Coast states, with approximately six human cases
and 200 horse cases per year. Florida has had more human and horse cases than any other state, with an average of 1-2 human and 70 horse cases per year, respectively (FDOH unpublished data).

EEEV has been shown to infect horses, humans, birds and other animals.\textsuperscript{1, 4} The virus is maintained in an enzootic cycle between ornithophilic mosquitoes and avian reservoir hosts.\textsuperscript{1, 5} Most studies of EEEV have concentrated upon foci in the northern parts of the US. In the northeastern states, transmission is seasonal, peaking in the late summer months.\textsuperscript{1} Passerine birds are considered to be the major reservoir hosts for EEEV in the northeastern US, with enzootic transmission between avian hosts mediated by ornithophilic \textit{Culiseta melanura} mosquitoes, particularly in freshwater swamp foci.\textsuperscript{1, 5, 6} Other mosquito species which feed upon mammals and birds, including \textit{Aedes vexans}, \textit{Aedes sollicitans}, and \textit{Coquillettidia perturbans}, have been implicated as bridge vectors, transmitting EEEV from birds to horses and humans.\textsuperscript{7, 8, 9} In the southeastern US, some evidence suggests that \textit{Culex erraticus} may play an important role both as an enzootic and bridge vector in habitats where \textit{Cs. melanura} is less common.\textsuperscript{10, 11} Shifts in feeding patterns of bridge vectors from feeding on avian to mammalian hosts during the transmission season may help to facilitate transmission to horses and humans.\textsuperscript{12, 13, 14, 15}

Most of the studies examining reservoir competence have focused on passerine birds.\textsuperscript{6, 16, 17, 18} Studies have indicated that permanent resident and summer resident birds have antibodies to EEEV more often than transient and winter resident birds\textsuperscript{6, 17, 19} and there is some evidence suggesting that migrating birds play a role in transportation of EEEV.\textsuperscript{20} Many of these studies focused on passerine birds; however, recent studies have indicated that wading birds (Ciconiiformes) are preferred hosts of potential bridge vectors of EEEV in the southeast.\textsuperscript{21, 22, 23, 24} Few studies have looked at the exposure of wading birds to EEEV; however, one
seroprevalence study conducted in Louisiana found that over 80% of yellow-crowned night herons (*Nyctanassa violacea*) were seropositive for EEEV, higher than any of the Passeriformes examined and virus was isolated from one nestling. Several other studies have implicated a wide variety of wading bird species as potential enzootic hosts for EEEV as well. In 1962, Herman reported 8 species of Ciconiiformes (great egret (*Ardea alba*), snowy egret (*Egretta thula*), black-crowned night heron (*Nycticorax nycticorax*), green heron (*Butorides virescens*), little blue heron (*Egretta caerulea*), tricolored heron (*Egretta tricolor*), yellow-crowned night heron, and white ibis (*Eudocimus albus*) as either being naturally or experimentally infected with EEEV. Experimentally infected wading and water birds have been shown to develop high enough viremias to infect mosquitoes. In addition, Ciconiiformes, particularly the night herons, have been implicated to some extent in many other arbovirus transmission cycles including West Nile virus, Saint Louis encephalitis virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus, Japanese encephalitis virus, and Murray Valley encephalitis virus.

Florida is unique among US states in that EEEV transmission occurs year-round. Furthermore, recent phylogenetic studies have suggested that Florida may serve as a reservoir for EEEV; the virus may periodically be introduced from Florida to the northeastern US, where it locally amplifies, overwinters, and can remain stable for several years. If the hypothesis that Florida serves as a reservoir for EEEV for the rest of the country is correct, then transmission during the winter months in Florida, when vector densities are low, may represent a particularly vulnerable point in the viral life cycle. The present study was undertaken to investigate winter ecology of EEEV transmission with respect to relative abundance, virus
infection, and host use of potential vector mosquito species, focusing on wading birds as potential amplification and dissemination hosts.

**Materials and Methods**

**Study Sites**

Field work was conducted at three county parks in Hillsborough County, Florida. Lettuce Lake Park (28°4'33.875"N, 82°22'35.837"W) is a 240-acre site along the Hillsborough River, with more than half of the park consisting of a hardwood and cypress swamp and the remainder consisting of hardwood hammocks and pine flatwoods. The park is named after a shallow dead-end offshoot of the river which is dominated by water lettuce (*Pistia stratiotes*), a floating aquatic plant. The second site, John B. Sargeant Park (28°4'57.793"N, 82°17'10.991W), is a 23-acre site also along the Hillsborough River, dominated by cypress and hardwood swamps. The third site, Eureka Springs Park (28°0'22.928"N, 82°20'41.529"W), is a 31-acre park with a floodplain forest of maple, cypress, and tupelo.

**Mosquito Collection**

The mosquito population at each site (park) was sampled weekly during winter months using three methods: resting shelters, carbon dioxide-baited light traps and vegetation sweeps. Resting mosquitoes were collected from wire frame shelters that served as artificial resting sites. 47 Six resting shelters were sampled at each site, spaced approximately 50 meters apart along designated trails. Mosquitoes were aspirated during morning hours (0800-1030h) from the resting stations using a modified Dustbuster ® and a piece of white corrugated plastic cardboard with a 10 cm diameter hole cut in it. 48 Mosquitoes resting in vegetation were also sampled during morning hours (0800-1030h) with vegetation sweeps. Low-growing herbaceous vegetation was swept using a heavy-duty sweep net along two predetermined paths and contents of the net.
vacuumed. Host-seeking mosquitoes were sampled using two CO$_2$–baited light traps at each site. Traps were set shortly before dusk, and retrieved the following morning, at which time resting mosquitoes were collected. Samples were collected weekly at Lettuce Lake Park from January through March 2012 and December through March 2013. Samples were collected weekly from the other two sites from December 2012 through March 2013. Mosquitoes were also collected from artificial resting shelters at Lettuce Lake Park from June through August 2011 for comparison. Field-collected mosquitoes were returned to the laboratory for identification, pool-screening, and blood meal analysis.

**Detection of Virus in Mosquito Pools**

Mosquitoes were sorted by species, collection date, and collection site into pools of 50 individuals or fewer. A copper BB and 1 ml of BFD (biological field diluent; 90% minimum essential medium with Hanks’ salts, 10% fetal bovine serum, 200 U/ml penicillin, 200 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 50 µg/ml kanamycin) were added to each pool and mosquitoes were homogenized using a high-speed mechanical homogenizer (TissueLyser; Qiagen, Valencia, CA). RNA was prepared from 140 µL of the supernatant using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s conditions. The Qiacube platform (Qiagen, Valencia, CA) was used to automate RNA extraction and the isolated RNA (eluted into 60 µl of nuclease free water) was stored at -80°C. Real time RT-PCR was then conducted using the iScript one step RT-PCR kit for probes (Bio-Rad, Hercules, CA) according to the manufacturer’s protocol. The primers and reaction conditions used to detect EEEV RNA were those recommended by Lambert and others,$^{49}$ with the exception that reactions were performed in a final volume of 25 µL, and used 5 µL of the RNA template. Samples producing a signal at a Ct value of 37 or below were considered putatively positive.
RNA samples found to be putatively positive in the initial assay were subjected to the confirmatory qRT-PCR assay provided by the Centers for Disease Control and Prevention to state Department of Health Laboratories conducting arboviral surveillance activities. The confirmatory assay employed two primers (5' ACCTTGCTGACGACCAGGTC 3' and 5' GTTGTGCTGCTCAATCCA 3') that produced an amplicon spanning positions 9428-9497 in the EEEV genome. The 5’ 6-FAM, 3’ BHQ1a-Q probe used in this assay contained the sequence 5’ CTTGGAAGTGATGCAAATCCAACTCGACA 3’, spanning positions 9449-9477 in the genome. Samples were only considered to be positive if they produced a detectable amplicon in both assays.

Virus isolation was attempted from all confirmed qRT-PCR positive samples by inoculating individual T-25 flasks of confluent Vero cell cultures with 1 mL of filtered mosquito pool supernatant. Flasks were incubated for two hours at 37°C, with gentle rocking every 15 minutes. After the incubation, 9 mL of maintenance media (1× Earle's minimal essential medium, 2% fetal bovine serum, 200 U/mL penicillin, 200 µg/mL streptomycin, 2.5 µg/mL amphotericin B) were added to each flask. Cells were then monitored daily for cytopathic effect (CPE).

**Blood Meal Analysis**

Individual blood engorged female mosquitoes were homogenized in 200 µL DNAzol reagent (Molecular Research Center Inc., Cincinnati, OH) using a disposable plastic pestle. Samples were incubated for ten minutes at room temperature, centrifuged and the supernatant transferred to a new tube. In order to precipitate the DNA, 80 µl of isopropanol was added. The solution was then mixed and incubated for five minutes at room temperature. The sediment was precipitated by centrifugation at 4,000 xg for ten minutes. Following centrifugation, the pellet
was washed twice with 1 ml of 75% ethanol and the DNA dissolved with 50 µl of Tris-EDTA buffer (Tris-EDTA, pH 8.0, Boston Bioproducts, Ashland, MA). Isolated DNA was stored at -80°C until further testing.

The identification of the blood meals from the extracted DNA employed two polymerase chain reaction (PCR)-based assays. The initial nested PCR used a set of universal vertebrate primers targeting cytochrome B. The first PCR reaction used the following primers: 5′-CCCCTCAGAATGATATTGTCCCTCA-3′ and 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and followed the reaction conditions outlined by Hassan and co-workers. The sequences of the forward primer for the nested reaction was 5′-TCWRCHTGATGAAACTTCGG-3′ where W = A or T, R = A or G, and H = A, C or T. The non-coding primer used was a mixture of four primers with the following sequences: 5′-ACRAARGCRGTGGCTATTAG-3′, 5′-ACRAAGGCAGTKGCTATAAG-3′, 5′-ACGAARGCRGTTGCYATGAG-3′, and 5′-ACGAAGGCMGTKGCTATTAG-3′ where K = G or T, Y = C or T, and M = A or C. Reaction conditions were as previously described.

The second PCR assay used to identify blood meals used a universal vertebrate primer set targeting 16S rRNA. Primers used in the PCR were those of Kitano and co-workers and were as follows: 5′-GCCTGTTTACCAAAAAACATCAC-3′ and 5′-CTCCATAGGGTCTTTCTCGTCTT-3′. Reaction conditions were the same as those described previously. Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and were then sent to the Eurofins MWG Operon sequencing facility (Huntsville, AL) for analysis. Sequences were entered into the NCBI BLAST database for identification, and only those sequences with a match percentage ≥ 95% were accepted as belonging to the identified blood meal source.
**Avian Surveys**

Wading birds were counted through visual searches of the study areas (mosquito collecting habitats) from elevated boardwalks maintained by The Parks, Recreation and Conservation Department of Hillsborough County at each of the parks. Wading birds were counted for each occasion that light traps were set (dusk) and again the following morning.

**Data Analysis**

In order to characterize the relative utilization of wading bird hosts as a blood source, forage ratios were calculated for each water bird species. Forage ratios were calculated by dividing the percentage of blood meals obtained by *Cx. erraticus* for each host species of interest by the percentage that the species represented in the water bird community. The maximum count for each bird for each sampling day (dusk or dawn) was used for calculating forage ratios. Abundance of some species of wading and water birds are difficult to estimate, however, increasing number of visits has been shown to yield significant increases in cumulative species richness in wetland areas. In addition, combining morning and evening samplings increases the overall detection probability for these species. We calculated 95% confidence intervals for forage ratios using the formula 95% CI = (forage ratio ± 1.96*standard error). The standard error (SE) was calculated using the formula $SE = \sqrt{\frac{o(1-o)}{u^2p^2}}$, where $o =$ the proportion of blood meals from a given source, $u =$ the total number of blood meals, and $p =$ the proportion of available host community comprised of species of interest. To account for variations in bird abundances over the season, the average monthly relative abundance was used in the calculations. Avian species that were found to be fed upon by mosquitoes, but not detected in the wading bird surveys, were assigned a value of 1 individual present for calculation of avian relative abundance and forage ratios. Forage ratios were not calculated for avian species for...
which no blood meals were detected but which were identified in the point counts. Forage ratios were considered to be statistically significant when their 95% confidence interval excluded one.  

Results

Mosquito Collections

A total of 12,260 mosquitoes representing 16 species were collected from the three sites over 116 trap nights during the winter months. *Culex erraticus* was the species with the highest abundance at all three sites, representing 58.0% of mosquitoes at Lettuce Lake Park, 41.9% at John B. Sargeant Park, and 33.3% at Eureka Springs Park (Figure 2.1). *Uranotaenia sapphirina* was the second most abundant species at Lettuce Lake Park, representing 12.6% of the mosquitoes collected. The remaining mosquito species at Lettuce Lake Park were collected at low abundance, with each species representing 10% or less of the collected mosquitoes at the site (Figure 2.1, Panel A). At John B. Sargeant Park, *Culex nigripalpus* and *Anopheles quadrimaculatus* were the next most abundant species, representing 21.3% and 19.9%, respectively (Figure 2.1, Panel B). The remaining mosquito species at John B. Sargeant Park were collected at low abundance, with each species representing less than 10% of the collected mosquitoes at the site. At Eureka Springs Park, *Culex nigripalpus* and *Anopheles crucians* were the next most abundant species, representing 32.4% and 24.0%, respectively (Figure 2.1, Panel C). *Culiseta melanura* was present at low numbers at all three study sites, representing less than 5% of the collections. Other species collected in low numbers at the three sites included *Culex territans, Culex peccator, Aedes infirmatus, Anopheles perplexens, Coquillettidia perturbans, Culex coronator, Culex peccator, Culex salinarius, Mansonia titillans, Uranotaenia lowii,* and
Wyeomyia mitchellii. Each of these species represented less than 2% of the collections from the sites or was not present at all sites.

Overall, mosquito abundances were higher in the second year of the study (December 2012-March 2013 winter season) than in the first year (January-March 2012 season) (Fig. 2, panels A and B). In general, abundance of host-seeking females was greatest in January in both years (Figure 2.2, Panels A and B), with few mosquitoes collected in weeks leading up to the end of the collection season in March. The one exception to this pattern was a peak of An. crucians that was observed in March, 2012 (Figure 2.2, Panel B). Culex erraticus was abundant during both winters, and was the only mosquito species collected during every month of sampling. In January of 2013, abundance of this mosquito was greater than any point during the entire study, with an average of 313.8 females collected per trap night the second week of January. Culiseta melanura was present, although not particularly abundant, throughout both winters.

Quantitative RT-PCR assays to detect EEEV were conducted on all non-engorged mosquitoes collected during both winter seasons. Of 455 total mosquito pools tested for EEEV, two pools were confirmed positive for EEEV; a pool of Cs. melanura collected at Lettuce Lake Park in February 2012 and a pool of An. quadrimaculatus collected at John B. Sargeant Park in February 2013. The overall seasonal minimum infection rate (MIR) was 22.2 for Cs. melanura and 1.54 for An. quadrimaculatus, while the monthly MIRs were 58.82 for Cs. melanura and 7.19 for An. quadrimaculatus. Attempts to culture virus from both isolates were not successful.

**Blood Meal Analysis - Winter Months**

A total of 724 blood-engorged mosquitoes representing eight species were collected during the two winter sampling periods (Table 2.1). Of these, 701 (96.8%) blood meals were successfully identified to the host species level. The most commonly collected blood-fed species
was *Cx. erraticus* with 500 blood meals identified. The percentages of blood meals identified by host class varied among the mosquito species. Birds were the predominant winter hosts for *Cx. erraticus, Cx. nigripalpus, Cx. peccator,* and *Cs. melanura. Culiseta melanura* had the highest proportion of avian blood meals (88.3%) among the eight mosquito species. For *An. crucians, An. perplexens,* and *An. quadrivittatus,* the majority of blood meals were from mammalian hosts; however, these species also fed to some extent on avian hosts. Amphibians were the main hosts for *Cx. territans. Culex territans* and *Cx. erraticus* were the only mosquito species found to feed upon all four host classes. Reptilian blood meals were mainly derived from alligators (*Alligator mississippiensis*) and the green anole (*Anolis carolinensis*) while amphibian hosts included the green tree frog (*Hyla cinerea*), Southern leopard frog (*Lithobates sphenoecephalus*), Cuban tree frog (*Osteopilus septentrionalis*), and pine woods tree frog (*Hyla femoralis*).

All eight species of mosquitoes fed upon both avian and mammalian hosts. A total of nine mammalian species were identified as hosts (Table 2.2). White tailed deer (*Odocoileus virginianus*) was a major host among mammals, representing more than half of the mammal-derived blood meals for *Cx. erraticus* (54.9%), *An. crucians* (63.2%) and *An. quadrivittatus* (51.7%). Humans (*Homo sapiens*) also represented important hosts, making up 29.1% of *Cx. erraticus* mammalian-derived blood meals, as well as 6/7 (85.7%) of the mammal-derived blood meals of *Cs. melanura*.

A total of 35 avian species were identified as hosts for the wintertime mosquito community during this study (Table 2.3). The vast majority of the avian blood meals came from wading birds. Seven of eight mosquito species fed upon wading birds to some extent. The only species not found to feed on wading birds, *An. perplexens,* had a single bird-derived blood meal.
(of seven total) from the Eastern phoebe (Sayornis phoebe). Anopheles crucians and An. quadriramaculatus took 13.6% and 14.7% of total blood meals from avian hosts, respectively. Wading birds were major avian hosts for Cx. erraticus and Cs. melanura, comprising 82.3% and 39.6%, respectively, of the total avian-derived blood meals for these species. Culex erraticus fed on 20 different bird species, 12 of which were water birds. The four most commonly fed upon birds were represented by three species of wading birds (black-crowned night heron, yellow-crowned night heron, and great blue heron (Ardea herodias)) and one species of water bird (anhinga (Anhinga anhinga)). Half (50.2%) of all avian-derived blood meals of Cx. erraticus came from night herons (black-crowned and yellow-crowned). Culiseta melanura fed on 21 bird species (7 species of water birds and 11 species of passerine birds). Just over half (50.9%) of avian-derived blood meals of Cs. melanura were from passerine birds, with the northern cardinal (Cardinalis cardinalis) contributing a quarter 24.5% of the total avian meals. By contrast, the northern cardinal represented just two (of 305 total) Cx. erraticus avian blood meals.

Host Utilization by Culex erraticus

For Lettuce Lake Park (Fig. 3, panel A), the forage ratios of Cx. erraticus for anhinga, black-crowned night heron, great blue heron, wood stork (Mycteria americana), and yellow-crowned night heron were significantly greater than one, indicating that these species were fed upon more frequently than would be expected based upon their relative abundance. These forage ratios ranged from 2.065 (wood stork) to 7.597 (black-crowned night heron). Green heron, limpkin (Aramus guarauna), pied-billed grebe (Podilymbus podiceps), and Wilson’s snipe (Gallinago delicata) also had forage ratios greater than one, but these were not statistically significant due in part to their large 95% confidence intervals. The Muscovy duck (Cairina moschata) and white ibis were the only species with forage ratios significantly less than one, and
were therefore underutilized as hosts. At John B. Sargeant Park (Fig. 3, panel B), forage ratios for the anhinga, great blue heron, and green heron were all significantly greater than one. Too few avian blood meals were identified from Eureka Springs Park (five total) to calculate realistic forage ratios. However, all 5 *Cx. erraticus* avian blood meals from this site were from wading birds, even though few wading birds were recorded at this site. Several species of birds were not present in the blood meals but were present in the avian survey; however this varied by location (Table 2.4). Conversely, there were also several species of birds that were not present in the avian survey but were present in the blood meals. Both the roseate spoonbill and the snowy egret were present at multiple sites but were not detected in any mosquito blood meals.

**Host Utilization - Summer and Winter Comparisons**

During the summer months of 2011, 145 blood-engorged *Cx. erraticus* females were collected from Lettuce Lake Park and the blood-meal source of 138 (95.2%) of these were identified to the species level. *Culex erraticus* fed upon 7 species of mammals, 13 species of birds, one species of amphibians, and 3 species of reptiles during the summer months (Fig. 4). Many of these blood meals came from mammals (44.9%), with the vast majority of the total blood meals being from white tailed deer (18.1%) and humans (21.7%). Additional mammalian blood meals included the bobcat (*Lynx rufus*), brown rat (*Rattus norvegicus*), dog (*Canis lupus familiaris*), raccoon (*Procyon lotor*), and wild boar (*Sus scrofa*), each of which made up less than 5% of the total blood meals. Alligators made up a large portion (17.4%) of the blood meals as well. The remaining reptilian blood meals came from brown water snake (*Nerodia taxispilota*, n=1) and pond slider turtle (*Trachemys scripta*, n=1). A total of 34.7% of *Cx. erraticus* blood meals came from avian hosts during the summer months, in contrast to the winter months, when avian hosts made up 61.0% meals. Five species of wading birds (black-crowned night heron,
great blue heron, limpkin, little blue heron, and yellow-crowned night heron) and two species of water birds (anhinga and pied-billed grebe) were each fed upon less than 10% of the time in the summer months. The only passerine bird species found to be fed upon was northern cardinal (2.9%). Additional avian species included barred owl (Strix varia), chicken (Gallus gallus domesticus), great horned owl (Bubo virginianus), osprey (Pandion haliaetus), and wild turkey (Meleagris gallopavo), which were all fed upon in small numbers.

Discussion

This field investigation found that vectors of EEEV were present and often abundant during the winter, that wading birds were important hosts for Cs. melanura and Cx. erraticus during the winter, and that EEEV-infected mosquitoes are actively host-seeking during the winter at wetlands in peninsular Florida. EEEV was detected in mosquitoes in Hillsborough County, Florida twice during winter months of the study. During the months of December through March in the years 2005-2010, 126 EEEV sentinel chicken seroconversions and 31 EEEV horse cases occurred in Florida (FDOH unpublished data). Winter transmission of EEEV in Hillsborough County has also been documented, with sentinel chicken seroconversions occurring during the study period, as well as one human case reported in March, 2013 (FDOH unpublished data). Although no sentinel chickens seroconverted at the Lettuce Lake Park sampling site during the study period, EEEV activity has been noted in the sentinel chickens maintained at this location in the past (FDOH unpublished data).

Both EEEV positive mosquito pools were from collections made during February, when mosquito abundance was relatively low. Both of the positive mosquito species (Cs. melanura and An. quadrivaculatus) have been shown to be competent vectors of EEEV, suggesting that these isolations represented evidence for ongoing winter transmission at the site.\textsuperscript{7,62,63,64}
*Culex erraticus* was the most abundant mosquito at these wetland sites during the winter months, a finding that corroborates those of other studies conducted in the southeastern US.\(^{10, 65, 66}\) While EEEV was not detected in *Cx. erraticus* pools during this study, this mosquito is a suspected bridge vector for EEEV, and has been shown to be a competent vector for the virus.\(^7\) Isolations of EEEV from *Cx. erraticus* mosquito pools in Florida have been demonstrated in the past.\(^{67, 68}\) Several studies have also found EEEV circulating during patterns of high *Cx. erraticus* and low *Cs. melanura* abundances.\(^{10}\) Theoretical models used for other arboviruses have indicated that having multiple vector species improves long-term virus persistence and species that are active in the winter could enable virus persistence\(^{69}\) until another vector becomes active in spring.\(^{70}\)

All eight blood fed mosquito species collected during the winter months fed on mammalian hosts to some extent in this study. The three blood-fed *Anopheles* species collected in this study, *An. crucians*, *An. quadrimaculatus*, and *An. perplexens*, fed more upon mammals than any other host class. The propensity for feeding on mammalian hosts shown by these species is well established,\(^{65, 71, 72, 73}\) though this study to our knowledge is the first to identify hosts for *An. perplexens* in Florida. The main mammalian host for *An. quadrimaculatus* and *An. crucians* as well as *Cx. erraticus* was the white tailed deer. White tailed deer are frequently exposed to EEEV in the wild\(^{29, 74}\) and several cases of clinical EEE have also been reported in this species.\(^{75, 76}\) The role that white tailed deer play in the transmission cycle of EEEV, however, remains unclear.

Another mammal commonly fed upon was humans. The relatively high preponderance of human blood meals seen in *Cx. erraticus* (29.1% of mammalian derived blood meals) and the 6 (10%) seen in *Cs. melanura* samples highlights the threat that these species may directly pose to
humans, as bridge vectors of EEEV. Although *Cs. melanura* is often considered to be an ornithophilic mosquito, the occasional feeding of *Cs. melanura* on humans has been documented previously.\textsuperscript{71,77}

The most commonly fed upon host class overall were avian hosts. All eight blood fed mosquito species fed on birds during the winter months, even those species that are known to feed predominantly on mammals (*An. crucians* and *An. quadrivittatus*) or reptiles and amphibians (*Cx. territans* and *Cx. peccator*).\textsuperscript{52,78} The few avian blood meals for these species were mainly represented by wading or water bird species. Interestingly, *An. quadrivittatus* s.l., one of the mosquito species positive for EEEV during this study, fed upon a wide variety of birds, including anhinga, raptors (barred owl and black vulture) and wading birds (wood stork and yellow-crowned night heron).

The enzootic vector of EEEV, *Culiseta melanura*, also fed predominantly on birds. Previous studies have found that this ornithophilic species feeds predominantly on passerine birds.\textsuperscript{77,79} In this study, the majority of females fed upon passerine birds (50.9%), but a relatively large percentage also fed on wading birds during the winter months (39.6%). Other studies have shown *Cs. melanura* feeding on wading birds, but to a smaller extent, representing around 7-15% of blood meals.\textsuperscript{22} It has been shown in other studies in Florida, however, that *Cs. melanura* may feed on Ciconiiformes at greater levels than would be expected from their relative abundance.\textsuperscript{79} No blood engorged females of *Cs. melanura* were collected during the summer months, so no conclusions could be made between host utilization between the two seasons. Nonetheless, our finding that *Cs. melanura* took a large fraction of total bloodmeals (nearly 40%) from wading birds in the winter is of interest, since this mosquito is considered the primary enzootic vector of EEEV in North America.
*Culex erraticus* fed upon all four host classes; however, the majority of females fed upon avian hosts. This catholic feeding pattern of *Cx. erraticus* has also been shown in other studies. The main avian hosts utilized by *Cx. erraticus* in this study were wading and water birds, representing 82.3% and 15.1% of avian blood meals, respectively. Other studies in the southeastern US conducted during different times of the year have shown a strong preference of *Cx. erraticus* for water/wading birds. A similar preference for herons, especially black-crowned night herons, was recently seen in Colombia as well. In contrast, passerine birds were fed upon to a much smaller extent by *Cx. erraticus*. In addition, a statistically significant preference by *Cx. erraticus* for several species of water and wading birds was seen, including the anhinga, black-crowned night heron, great blue heron, green heron, wood stork, and yellow-crowned night heron. Several other wading birds had an increased preference but the preference did not reach statistical significance. This was due to a lack of power resulting from the bird species in question not being observed during the avian surveys or due to low numbers of blood meals identified from these species.

The stalking and nesting behaviors exhibited by many species of wading birds may influence their ability to serve as a potential enzootic hosts for EEEV. Night herons stand still for long periods of time as they forage for food and have decreased anti-mosquito behavior compared to other species including other Ciconiiformes, allowing many mosquitoes to feed upon them. However, in these studies, the authors concluded that anti-mosquito behavior couldn’t explain all the variation in feeding preference by mosquitoes, suggesting an innate preference for these species.

While birds were the most commonly fed upon host class for *Cx. erraticus* during the winter months, mammals were more commonly fed upon during the summer. Almost 45% of
females fed upon mammalian hosts in the summer months, particularly humans (21.7% of total
blood meals). Humans only made up 10.2% of the total blood meals in the winter months,
indicating an increased risk of exposure for mammalian species to EEEV during the summer.
These results indicate a shift in feeding behavior occurring somewhere between the winter and
summer months. Such a biphasic pattern of feeding has also been seen in studies of other
mosquito species, and has been hypothesized to play an important role in arboviral transmission
and amplification.\textsuperscript{12, 13, 14, 15} One reason for this may be an increased abundance of wading birds
at our study sites in the winter months due to the inmigration of these species, which augment
the year round resident populations.\textsuperscript{87, 88} However, the four most commonly fed upon avian
species during the winter months were still among the most often fed upon avian hosts in the
summer, although they were targeted to a lesser extent than in the winter, suggesting that these
species remain popular hosts year round, despite the seasonal fluctuations in their numbers.

In summary, the data presented above demonstrates a preference for feeding on water
birds and wading birds among many mosquito species, notably \textit{Cx. erraticus} and \textit{Cs. melanura},
during the winter months. Given that previous studies have shown these to be competent
enzootic hosts for EEEV, it is possible that these species may play a role in maintaining EEEV
transmission during the winter months in Florida, and perhaps in disseminating the virus to the
northeastern states during their spring migration. Further research will be needed to investigate
the role of wading birds in these processes.
### Tables and Figures

**Table 2.1** Proportion of blood meals taken from different host classes during the winter months

<table>
<thead>
<tr>
<th>Species</th>
<th>ID/Tested</th>
<th>Avian</th>
<th>Mammalian</th>
<th>Reptile</th>
<th>Amphibian</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anopheles crucians</em></td>
<td>22/22</td>
<td>13.6 ± 14.3</td>
<td>86.4 ± 14.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Anopheles perplexens</em></td>
<td>7/7</td>
<td>14.3</td>
<td>85.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Anopheles quadrimaculatus</em></td>
<td>34/34</td>
<td>14.7 ± 11.9</td>
<td>85.3 ± 11.9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Culex erraticus</em></td>
<td>500/518</td>
<td>61.0 ± 4.3</td>
<td>35.0 ± 4.2</td>
<td>3.2 ± 1.5</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td><em>Culex nigripalpus</em></td>
<td>40/40</td>
<td>75.0 ± 13.4</td>
<td>20.0 ± 12.4</td>
<td>0</td>
<td>5.0 ± 6.8</td>
</tr>
<tr>
<td><em>Culex peccator</em></td>
<td>7/8</td>
<td>71.4</td>
<td>28.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>Culex territans</em></td>
<td>31/35</td>
<td>9.7 ± 10.4</td>
<td>12.9 ± 11.8</td>
<td>6.4 ± 8.6</td>
<td>71.0 ± 16.0</td>
</tr>
<tr>
<td><em>Culiseta melanura</em></td>
<td>60/60</td>
<td>88.3 ± 8.1</td>
<td>11.7 ± 8.1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Only mosquito species for which the number of blood meals identified was greater than 5 are shown. 95% confidence intervals are provided for species where the number of samples identified was greater than or equal to 20.*
Table 2.2 Blood meals from mammalian hosts during the winter months

<table>
<thead>
<tr>
<th>Host species</th>
<th>Anopheles crucians (n=19/22)</th>
<th>Anopheles perplexens (n=6/7)</th>
<th>Anopheles quadrimaculatus (n=29/34)</th>
<th>Culex erraticus (n=175/500)</th>
<th>Culex nigripalpus (n=8/40)</th>
<th>Culex peccator (n=2/7)</th>
<th>Culex territans (n=4/31)</th>
<th>Culiseta melanura (n=7/60)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow (Bos taurus)</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>9</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dog (Canis lupus familiaris)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Eastern cottontail rabbit (Sylvilagus floridanus)</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human (Homo sapiens)</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>51</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Marsh rabbit (Sylvilagus palustris)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Raccoon (Procyon lotor)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Virginia opossum (Didelphis virginiana)</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>White tailed deer (Odocoileus virginianus)</td>
<td>12</td>
<td>0</td>
<td>15</td>
<td>96</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Wild boar (Sus scrofa)</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The numerator under each mosquito species name indicates the number of blood meals from mammals, while the denominator indicates the total number of blood meals identified (from all host classes) for that mosquito species.
### Table 2.3 Number of blood meals from avian hosts during the winter months

<table>
<thead>
<tr>
<th>Host species</th>
<th>Anopheles crucians (n=3/22)</th>
<th>Anopheles perplexens (n=1/7)</th>
<th>Anopheles quadrimaculatus (n=5/34)</th>
<th>Culex erraticus (n=305/500)</th>
<th>Culex nigripalpus (n=30/40)</th>
<th>Culex peccator (n=5/7)</th>
<th>Culex territans (n=3/31)</th>
<th>Culiseta melanura (n=53/60)</th>
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</thead>
<tbody>
<tr>
<td>American bittern (<em>Botaurus lentiginosus</em>)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Anhinga (<em>Anhinga anhinga</em>)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>41</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Black-crowned night heron (<em>Nycticorax nycticorax</em>)</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>110</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Eastern phoebe (<em>Sayornis phoebe</em>)</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Great blue heron (<em>Ardea herodias</em>)</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>33</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>1</td>
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<td>Great egret (<em>Ardea alba</em>)</td>
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<td>23</td>
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<td>Green heron (<em>Butorides virescens</em>)</td>
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<td>Limpkin (<em>Aramus guarauna</em>)</td>
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<td>Little blue heron (<em>Egretta caerulea</em>)</td>
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<td>Muscovy duck (<em>Cairina moschata</em>)</td>
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<td>2</td>
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<td>0</td>
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</tr>
<tr>
<td>Northern cardinal (<em>Cardinalis cardinalis</em>)</td>
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<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>Northern parula (<em>Parula Americana</em>)</td>
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<tr>
<td>Pied-billed grebe (<em>Podilymbus podiceps</em>)</td>
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<tr>
<td>Tennessee warbler (<em>Vermivora peregrina</em>)</td>
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<td>Tufted titmouse (<em>Baeolophus bicolor</em>)</td>
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<td>Turkey vulture (<em>Cathartes aura</em>)</td>
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<tr>
<td>White ibis (<em>Eudocimus albus</em>)</td>
<td>0</td>
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<td>0</td>
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<tr>
<td>Wood stork (<em>Mycteria americana</em>)</td>
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<td>28</td>
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<td>Yellow-crowned night heron (<em>Nyctanassa violacea</em>)</td>
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<td>43</td>
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<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Other Passeriformes species (n=8)</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Other avian species (n=8)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

*Only avian species with more than one mosquito blood meal identified are listed. Passeriformes species not included in the table were Carolina wren (*Thryothorus ludovicianus*) and Florida scrub jay (*Aphelocoma coerulescens*), for *Cx. erraticus*; blue jay (*Cyanocitta cristata*), hermit thrush (*Catharus guttatus*), house wren (*Troglodytes aedon*), loggerhead shrike (*Lanius ludovicianus*), pine warbler (*Dendroica pinus*), and white breasted nuthatch (*Sitta carolinensis*) for *Culex nigripalpus*; great horned owl (*Bubo virginianus*) and Wilson’s snipe (*Gallinago delicata*) for *Culex erraticus*; black vulture (*Coragyps atratus*), mourning dove (*Zenaida macroura*), and osprey (*Pandion haliaetus*) for *Culex nigripalpus*; chicken (*Gallus gallus*), and wild turkey (*Meleagris gallopavo*) for *Culex melanura*. The numerator under each mosquito species name indicates the number of blood meals from birds, while the denominator indicates the total number of blood meals identified (from all host classes) for that mosquito species.*
Table 2.4 Average monthly water bird abundance and proportion of *Cx. erraticus* blood meals during the winter months

<table>
<thead>
<tr>
<th>Lettuce Lake Park</th>
<th>Average Monthly Relative Abundance (SD)</th>
<th>Blood Meal Proportion Avian ±CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anhinga</td>
<td>0.030 (0.009)</td>
<td>0.128 ± 0.039</td>
</tr>
<tr>
<td>Black-crowned night heron</td>
<td>0.051 (0.036)</td>
<td>0.390 ± 0.057</td>
</tr>
<tr>
<td>Double crested cormorant</td>
<td>0.007 (0.006)</td>
<td>0</td>
</tr>
<tr>
<td>Duck (Mottled, Muscovy, Whistling)</td>
<td>0.050 (0.032)</td>
<td>0.007 ± 0.010</td>
</tr>
<tr>
<td>Great blue heron</td>
<td>0.032 (0.019)</td>
<td>0.082 ± 0.032</td>
</tr>
<tr>
<td>Great egret</td>
<td>0.073 (0.021)</td>
<td>0.078 ± 0.031</td>
</tr>
<tr>
<td>Green heron</td>
<td>0</td>
<td>0.007 ± 0.010</td>
</tr>
<tr>
<td>Limpkin</td>
<td>0.010 (0.004)</td>
<td>0.021 ± 0.017</td>
</tr>
<tr>
<td>Little blue heron</td>
<td>0.020 (0.009)</td>
<td>0</td>
</tr>
<tr>
<td>Pied-billed grebe</td>
<td>0</td>
<td>0.007 ± 0.010</td>
</tr>
<tr>
<td>Roseate spoonbill</td>
<td>0.012 (0.015)</td>
<td>0</td>
</tr>
<tr>
<td>Snowy egret</td>
<td>0.004 (0.002)</td>
<td>0</td>
</tr>
<tr>
<td>White ibis</td>
<td>0.607 (0.113)</td>
<td>0.004 ± 0.007</td>
</tr>
<tr>
<td>Wilson’s snipe</td>
<td>0</td>
<td>0.004 ± 0.007</td>
</tr>
<tr>
<td>Wood stork</td>
<td>0.048 (0.041)</td>
<td>0.100 ± 0.035</td>
</tr>
<tr>
<td>Yellow-crowned night heron</td>
<td>0.056 (0.025)</td>
<td>0.145 ± 0.041</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>John B. Sargeant Park</th>
<th>Average Monthly Relative Abundance (SD)</th>
<th>Blood Meal Proportion Avian ±CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>American bittern</td>
<td>0</td>
<td>0.056 ± 0.106</td>
</tr>
<tr>
<td>Anhinga</td>
<td>0.060 (0.028)</td>
<td>0.288 ± 0.207</td>
</tr>
<tr>
<td>Great blue heron</td>
<td>0.029 (0.026)</td>
<td>0.389 ± 0.225</td>
</tr>
<tr>
<td>Great egret</td>
<td>0.054 (0.020)</td>
<td>0.056 ± 0.106</td>
</tr>
<tr>
<td>Green heron</td>
<td>0.001 (0.002)</td>
<td>0.222 ± 0.192</td>
</tr>
<tr>
<td>Limpkin</td>
<td>0.007 (0.010)</td>
<td>0</td>
</tr>
<tr>
<td>Little blue heron</td>
<td>0.067 (0.051)</td>
<td>0</td>
</tr>
<tr>
<td>Roseate spoonbill</td>
<td>0.001 (0.002)</td>
<td>0</td>
</tr>
<tr>
<td>Snowy egret</td>
<td>0.002 (0.002)</td>
<td>0</td>
</tr>
<tr>
<td>Tricolored heron</td>
<td>0.009 (0.009)</td>
<td>0</td>
</tr>
<tr>
<td>White ibis</td>
<td>0.748 (0.134)</td>
<td>0</td>
</tr>
<tr>
<td>Wood stork</td>
<td>0.016 (0.007)</td>
<td>0</td>
</tr>
<tr>
<td>Yellow-crowned night heron</td>
<td>0</td>
<td>0.400 ± 0.429</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eureka Springs Park</th>
<th>Average Monthly Relative Abundance (SD)</th>
<th>Blood Meal Proportion Avian ±CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great blue heron</td>
<td>0</td>
<td>0.600 ± 0.429</td>
</tr>
<tr>
<td>Great egret</td>
<td>0.599 (0.468)</td>
<td>0</td>
</tr>
<tr>
<td>Limpkin</td>
<td>0.028 (0.056)</td>
<td>0</td>
</tr>
<tr>
<td>White ibis</td>
<td>0.337 (0.398)</td>
<td>0</td>
</tr>
<tr>
<td>Wood stork</td>
<td>0.036 (0.071)</td>
<td>0</td>
</tr>
<tr>
<td>Yellow-crowned night heron</td>
<td>0</td>
<td>0.400 ± 0.429</td>
</tr>
</tbody>
</table>

*Relative abundance was calculated from the average monthly abundance by dividing the number of birds of that species by the total number of water birds that month. 95% confidence intervals are provided for blood meal proportions.*
Figure 2.1 Total relative abundance of mosquitoes (n=12,260) collected from Lettuce Lake Park (panel A), John B. Sargeant Park (panel B), and Eureka Springs Park (panel C) in Hillsborough County, FL during two winter collection periods (2012-2013).
Figure 2.2 Winter abundance of mosquitoes from 3 wetland parks in Hillsborough County, FL; January - March 2012 (panel A) and December, 2012 – March, 2013 (panel B). Asterisks indicate dates of EEEV-positive mosquito pools.
Figure 2.3 Forage ratios for water birds as hosts for *Cx. erraticus* from Lettuce Lake Park (panel A) and John B. Sargeant Park (panel B) during two winter collection periods (2012-2013). In each panel, the horizontal axis crosses at one, so that species with forage ratios above 1 are indicated as positively preferred and those less than 1 are negatively preferred. Asterisks indicate host species whose forage ratios were statistically different from 1.0.
Figure 2.4 Distribution of hosts fed upon by *Cx. erraticus* collected from Lettuce Lake Park, summer 2011. Numbers over bars indicate total number of blood meals identified for each host class.
References


CONCLUSION

In order to decrease transmission of eastern equine encephalitis virus (EEEV), it is important to know as much information about the transmission cycle as possible. The transmission cycle of this virus is complicated due to the multiple vectors and hosts, and not all aspects have been completely described. Since Florida is capable of year-round transmission of EEEV, the southeastern United States may serve as a reservoir for the virus for more northern locations. Recent phylogenetic studies have confirmed this, with Florida isolates clustering with isolated obtained from the northeastern United States. These studies also indicated that the virus is able to locally amplify, overwinter, and can remain stable in these locations for several years. How EEEV is able to migrate from Florida and how it persists during the winter in North America is not yet known. The first study contained herein has indicated that snakes have detectable levels of circulating EEEV viremia and may play a role as an overwintering host for the virus. The second study found that wading birds in Florida are preferred hosts in the winter for several known or suspected vectors of EEEV, and may play a role in the dispersal of the virus to more northern locales.

These studies, while providing us with more knowledge on the transmission and ecology of EEEV, are mainly an indication of potential areas of further study. Other studies have indicated that snakes are exposed to EEEV and this study was the first to isolate viral RNA from them as well. However, since this research was limited to Alabama, it would be important to test snakes for exposure to and infection with EEEV from other areas as well. If snakes can
serve as overwintering hosts for the virus, it would be especially important to repeat this study in
the northeast. Being able to isolate and culture the virus from naturally infected snakes is also
important as it would further provide strong evidence for the association and would allow for
genetic comparisons between EEEV isolates from other hosts. An association between potential
vectors of EEEV and snakes has also been shown, but many of these mosquito species are
suspected vectors of EEEV and the necessary vector competency studies need to be conducted.
These studies were also conducted in the southeast and should be repeated to see which mosquito
species are feeding on snakes in other locations. In addition, the timing of when mosquitoes feed
on snakes and when the snakes have high levels of viremia, particularly how viremia levels
change post-brumation, also needs to be investigated.

Laboratory studies have also indicated that snakes may serve as reservoir hosts for
EEEV, however, these studies do not indicate whether the snake species used were
picked based on past exposure studies or due to their ease of handling or access. This study
found that cottonmouth snakes were more often exposed at the study site than other species.
Future studies should focus on the susceptibility of cottonmouths to infection and how
temperature affects their levels of viremia. In addition, these studies have only looked at
infecting the snake with virus. In order to show that the virus can be transmitted between snake
and mosquitoes, it is also important to try to infect mosquitoes from an infected snake and vice
versa. Unfortunately, the use of cottonmouth snakes in these studies creates problems in terms of
both safety and regulations affecting venomous animals. Studies similar to the one conducted by
Hayes and co-workers, where infected snakes were left outside over the winter and tested for
EEEV the next spring, would also provide a lot of information in terms of the ability of snakes to
serve as an overwintering host. However, this type of study would also violate many legal and safety regulations.

For the second study, many of the same problems come up in terms of verifying that wading birds are involved in the potential dispersal and maintenance of EEEV. Laboratory studies have been conducted with several wading bird species in the past, and found that they have high enough viremia to infect mosquitoes, but none of these studies tried to infect mosquitoes from infected wading birds. It is also important to know how long high levels of viremia can last in these birds when considering them as potential dispersers of the virus. Unfortunately, little laboratory work can be done now due to legal regulations and many species may be endangered, threatened, or species of special concern, so these important studies may not be completed.

Studies have implicated a wide variety of wading bird species as potential hosts for EEEV. The majority of these studies occurred in the southeastern United States and other studies looking at exposure and infection rates in other locations needs to be conducted. While virus has been isolated from several species of wild wading birds, it would be beneficial to attempt further isolations from other locations as well. Studies have also indicated that wading birds are preferred hosts of potential bridge vectors of EEEV in the southeast. However, again, it would be important to determine if these same species are being fed upon by vectors of EEEV in the northeast. Many of these wading birds overwinter in Florida and other states in the southeast before returning to their nesting and post-breeding dispersal ranges. One way to help confirm that wading birds are involved with dispersal of the virus would be to tag wading birds, particularly those that have been implicated as preferred hosts such as black- and yellow-crowned night herons, with a GPS logger that tracks their location. You can then go
to these locations and see if the arrival of the wading birds contributes to or jumpstarts the enzootic transmission of EEEV. Due to the short period of high viremia for many of these birds, it is also possible that there are locations between the southeast and northeast that may serve as intermediate sites, and the GPS tracker would indicate these locations as well. Knowing where the birds migrate and when can help to determine locations to test mosquitoes for virus and blood meal hosts as well as testing birds for exposure to and infection with EEEV.

Knowing more about the transmission cycle and virus dispersal, maintenance, and overwintering is important when it comes to control of EEEV. Most mosquito control efforts tend to be focused towards the epizootic transmission of arboviruses that peaks in the summer as enzootic transmission is not always detectable and mosquito densities may be lower in earlier months. Mosquito control efforts could be made to coincide with the timing of bird migrations or the timing of snake brumation. Efforts could even be made to correspond with the timing of known winter transmission of EEEV, as seen in the second study that showed isolates of EEEV in February when mosquito densities were low. If transmission was reduced during the early season amplification of the virus, the level of arbovirus transmission could be reduced for the rest of the year.
References


APPENDIX

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