HOST PREFERENCE PROFILE IN AN AREA OF EASTERN EQUINE ENCEPHALITIS (EEE) VIRUS TRANSMISSION IN ALABAMA

by

ANA L. OLIVEIRA

SIBYLLE KRISTENSEN, CHAIR
CRAIG WILSON
CHARLES KATHOLI
ROBERT NOVAK
THOMAS UNNASCH

A DISSERTATION
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Doctor of Public Health
BIRMINGHAM, ALABAMA
2010
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ANA L. OLIVEIRA

PUBLIC HEALTH

ABSTRACT

Eastern Equine Encephalitis virus (EEEV) circulates in a mosquito-avian cycle (enzootic) within well-characterized environments. However, outbreaks in birds, horses and humans can occur (epizootic/epidemic transmission). EEEV is present throughout the Americas and the Caribbean, although some geographical differences in the ecology and epidemiology of the virus exist. The North American type of EEEV is associated with severe disease in both humans and horses, and is transmitted by the enzootic vector Culiseta melanura among birds in northern North America. The South American EEEV strain in horses can present similarly to the North American type, however, in humans, infection is rare and usually presents with asymptomatic or milder form of the disease. Culex melanoconion has been implicated as the enzootic vector and small mammals and birds may serve as EEEV hosts in South America. The epidemiology of EEEV in the southeastern USA might be similar to the epidemiology of EEEV in South America. Recent advances in blood meal analysis techniques permit that we look into patterns of host preference and in consequence into the relationships between hosts and possible vectors in areas of arboviral transmission. Temporal shifts among host classes in feeding patterns of the mosquito vectors are believed to be an important factor in arbovirus ecology. The presence of temporal changes in the host preference profile of mosquito vectors involved in Eastern Equine Encephalitis Virus (EEEV) transmission at a site at the Tuskegee National Forest (TNF), Alabama, has been investigated employing a dataset
containing a total of 1,583 identified blood meals from 90 different host species collected over six years. Runs test (a test of randomness) revealed that blood meals from the Great Blue Heron (GBH) and White-tailed Deer (WTD) (two of the most fed upon species) were not random across years; while meals derived from the Yellow-crowned Night Heron (another of the most frequently fed upon species) appeared to be randomly distributed. Tukey’s two-way ANOVA with 1 degree-of-freedom (test for interaction) showed that normalized blood meal counts for WTD and GBH were consistent from year to year. These data suggest that the temporal profile of feeding shifts for certain species exhibited a non-random pattern that was consistent from year to year, suggesting that some species might be preferentially fed upon while other species seemed to be more opportunistically targeted.
DEDICATION

To my family, for their unconditional love and support. I dedicate this work to my dear husband Lucio, and my wonderful children, Millena and Victor, for all their love and sacrifices during my time in graduate school. To my parents, who gave me life and love, and since early in my life, gave me the means for getting educated and fostered in me the interest for knowledge. Now, later in my life, even if from many miles away, they are still an inextinguishable source of inspiration and support. To all of my family and friends that always cheered me on throughout my life in every endeavor I ventured on without doubting for a minute I could succeed. To God, Who mercifully and lovingly makes all things possible.
ACKNOWLEDGMENTS

This work would not have been possible without the valuable guidance, inspiration, support, and encouragement of many people. I would like to thank my committee chair, Dr. Sibylle Kristensen, who always supported me and encouraged me to keep going even at the darkest moments. I am also indebted to Dr. Thomas Unnasch for his guidance before I worked in this degree (as my boss) and during this graduate program for the opportunity to work on the data collected by his research group as part of this dissertation. I would also like to thank Dr. Charles Katholi for his always patient and kind advice and enlightenment into the exciting world of Statistics. I own many thanks to Dr. Robert Novak for his suggestions and advice especially during our conversations about arbovirus, Entomology and Science in general. I am also grateful to Dr. Craig Wilson for his support and guidance through my years in Geographic Medicine and during this work. Dr. Julian Rayner, my most recent employer at UAB, also receives my appreciation for always being an incredibly supportive, nurturing and friendly boss, and to Dr. David Freedman for all his support and for the invitation and the opportunity to join his research group at UAB over 10 years ago. I am grateful too to Dr. Unnasch’s Auburn field team under the current leadership of Nathan Burkett-Cadena for the sample collection for the arbovirus project, and my dear friends from Dr. Unnasch’s lab staff, in special Hassan Hassan for all his hard work in the blood meal analysis.

I would also like to thank all other faculty members at the UAB School of Public
Health for the knowledge I gained through their lectures. I would like to especially mention former faculty members, Drs. Michael Maetz and Sten Vermund for all their kind and inspirational support, in and out of the classroom, throughout all these years since I arrived at Birmingham (Dr. Vermund) or decided to start a career in Public Health (Dr. Maetz and Dr. Vermund). I also would like to thank Gayla Watts and Kimberly Hawkins for all their assistance throughout these years I have been in the School of Public Health in my masters and doctoral programs. I would also like to acknowledge my co-workers at the Epidemiology Division of the Alabama Department of Public Health where I have the honor and the privilege of putting all the skills learned at the School of Public Health in practice everyday. Their commitment to Public Health and their professionalism, their support and trust in me, has inspired me and reassured me of my own passion and commitment to work in Public Health. As I approach the final steps into my terminal degree, I cannot forget where it all started. I am thankful to all my teachers since childhood who inspired, taught, and supported me. I own a special thanks to my teachers, mentors, and friends from college and graduate school (MS) at the Federal University of Pernambuco, Brazil, especially the ones at the Biochemistry and Biophysics department and my former mentors Drs. Valfrido Santana and Elizabeth Malagueno.

My family and friends have always had a paramount importance in my life and in any big or small accomplishments I have made in my life. My parents, sisters, cousins, aunts, uncles, grandma, grandpas, and friends who since my birth always believed in me and think naively and lovingly that I am one of the most intelligent people in the world. That kind of belief helped me to keep going even in difficult times, and made me strive to
work as hard as possible not to disappoint them. They are indeed the best cheerleader squad, the best support group, most efficient anti-depressant, and most potent energetic multivitamins anyone could wish for. To all my friends in Birmingham and the ones from the School of Public Health and throughout UAB (in special the fellow graduate students in many programs at UAB for all the listening and sharing), my sincere thanks -- you all made my time and my family’s time here in Birmingham especial, happier and memorable. Thank you all for the contributions you all made to my life in general and to this work.
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INTRODUCTION

Eastern Equine Encephalitis Virus (EEEV)

Arboviruses are arthropod–borne viral diseases, or diseases transmitted by arthropods (mosquitoes or ticks). Transmission occurs through the saliva of an infected mosquito during taking of a blood meal from a host [1]. There are many arboviruses of public health importance causing disease in humans and animals, and Eastern Equine Encephalitis virus (EEEV) is one of them.

EEEV is a positive-sense single-stranded RNA Alphavirus (Genus) of the Togaviridae family [1-6]. Alphaviruses are enveloped viruses [3, 5], and their genome is about 11-12 kb in length [3, 4, 7]. The 5’ end part of the genome encodes for the four non-structural proteins and other related proteins [4, 7]. The 3’ end of the genome encodes for the three structural proteins: capsid (protein C), and enveloped glycoproteins E1 and E2 (Figure 1) [4, 7].

Figure 1. Computer-generated model of the surface of an Alphavirus derived by cryoelectron microscopy. The spike-like structures on the virion surface are trimers composed of heterodimers of the virion surface glycoproteins E1 and E2. They are used by the virus to attach to susceptible animal cells. Image taken from http://www.cdc.gov/ncidod/dvbid/arbor/alphavir.htm
EEEV has two main strains, the North American and South American strains, based on antigenic variability exhibited in agglutination and neutralization assays [4, 7]. North American types of EEEV are more conserved, exhibiting around a 2% difference in their sequences [4, 7, 8], while South American strains are more diverse, differing by around 25% in their nucleotide sequence [7, 8]. North American strain (lineage I) circulates in North America and the Caribbean and the South American strain (lineages II, III, and IV) in countries of Central and South America [4, 7, 9, 10].

Transmission

EEEV transmission cycle has two types of hosts: birds and small mammals where the virus replicates at high levels (and therefore they can also function as reservoirs and/or virus amplifiers), and the so-called “dead-end” hosts where the virus does not replicate in high enough levels in their circulation to sustain further transmission. Dead-end hosts have no implication in the continuation of viral transmission but can present with symptoms of the viral infection (Figure 2) [1]. In North America, mosquitoes of the species *Culiseta melanura* have been implicated in transmission of EEEV between passerine birds (in what is called the enzootic cycle) [2, 4, 6, 7]. In South America, *Culex* mosquitoes have been implicated in EEEV enzootic transmission [2-4, 6, 7]. In South America, less is known about the epidemiology and ecology of EEEV due to the very low incidence of human cases [6, 7], but it is believed that small mammals and birds are the host for EEEV in that area [6, 7]. When other mosquitoes of less restricted feeding behavior are involved in EEEV transmission, transmission of EEEV from birds to
humans or other mammals such as horses can occur (which constitutes the epizootic transmission) [7].

Transmission of EEEV in temperate climates is seasonal (peaking in early Summer, late Fall) and in tropical areas seems to happen throughout the year [7]. Although the initiators of epizootic transmission of EEEV are not well known, it usually occurs in North America in a cyclical fashion with epidemics occurring every few years. That might reflect the fact that the virus needs high population of vectors for transmission to be initiated and maintained. In addition, once the number of susceptible hosts reaches a certain number, epizootic transmission stops [7]. EEEV transmission is very localized and occurs in and around swamp areas throughout the Americas and the Caribbean [11].

Figure 2. The arbovirus transmission cycle. Image taken from http://www.cdc.gov/ncidod/dvbid/arbor/schemat.pdf
Many theories have been suggested to explain how EEEV can be maintained in its enzootic foci. These theories usually relate to ways through which the virus could be introduced in the transmission area every season, or to how the virus could remain “dormant” in its foci and resurge in the next transmission season. One way for viruses to be introduced in new areas is by migratory birds. The role of this type of virus introduction in North America has been discounted in EEEV ecology by studies in some areas due to findings such as juvenile birds infected prior to evidence of mosquito positive pools in some areas [7], and resident birds other than migratory birds being found seropositive for the virus in other areas [10, 12]. In the Caribbean, however, it is thought that EEEV is introduced by migratory birds from North America [7]. Another possibility is that viruses could stay dormant in transmission areas between transmission seasons by means of over-wintering. One of the types of overwintering, transovarial transmission, occurs when the infected female lays infected eggs that stay dormant and develop into adult mosquitoes already infected at the beginning of the next transmission season. This has not been found in laboratory experiments looking for evidence of EEEV infected larvae and male mosquitoes [7]. Infected adult mosquitoes could also hibernate and be able to start viral transmission once the winter is over [13]. Another way of over-wintering is for the virus to stay through transmission seasons in host species at the local virus transmission foci [11]. Host classes involved in possible over-wintering could include bird, small mammals, and even amphibians and reptiles as it has been hypothesized to happen in transmission areas in Alabama [8, 14, 15]. It is possible that a combination of the hypothesis cited above or all of them can occur, each contributing more or less depending on the local conditions at a specific time in a transmission foci.
Pathology

EEEV infection presentation is non-specific (can have similar symptoms in many other arboviruses and other infectious and non-infectious conditions) and in humans and animals, viremia persists for 3-5 days [2]. Clinically, infection due to EEEV manifests with a range of symptoms from sub-clinical febrile illness to severe encephalitis [5, 8]. Initially EEEV infection has a systemic presentation where the virus replicates in peripheral sites. Symptoms of the systemic phase of infection include fever, chills, malaise, and myalgia. These systemic symptoms usually last 1-2 weeks and can either resolve or progress to encephalitis (symptoms include fever, headache, vomiting, seizure and coma) [8, 16]. Encephalitis can have an abrupt onset or appear after a few days of systemic disease, and death can occur after 2-10 days of onset of symptoms [8, 16]. The death rate in horses can reach 90% with 66% of survivors presenting with sequelae. In humans, mortality rate can reach 30%-50% with half of survivors presenting with neurological sequelae [16].

Neurological sequelae can vary in severity but are usually associated with increased mortality rates, estimated to be around 90% at nine years post-infection with only a 3% survival rate [8]. A serological study in New Jersey, during an outbreak of EEEV in 1959 estimated that the mean ratio of symptomatic to asymptomatic infections for EEEV was 1:23 (ranging from 1:16 to 1:23) [8, 17]. This ratio of symptomatic/asymptomatic infections may be higher in adults (1:40) [8].

There is no specific treatment for EEEV infection [2]. However studies in mice have investigated chemical compounds and their ability to inhibit disease caused by Togoviridae family viruses VEE and Semliki Forest including recombinant interferon
There is an experimental EEEV vaccine for humans recommended for laboratory technicians working with the virus, and a licensed vaccine for horses [8, 16].

Epidemiology

In the US, according to the CDC, 247 cases of EEEV in humans have been recorded from 1964-2008. The top five EEEV cases reporting states in the US during 1964-2008 are Florida (66 cases), Georgia (28 cases), Massachusetts (35 cases), New Jersey (20 cases), and Louisiana & North Carolina (16 cases each). In Alabama there have been only seven human cases reported from 1964-2008 (Figure 3).

![Figure 3. Geographical Distribution of human EEEV cases in the US from 1964-2008.](http://www.cdc.gov/ncidod/dvbid/arbor/pdf/EEE_Map.pdf)

Both sexes seem to be equally affected by EEEV infection [8]. The majority of infections in outbreak situations are observed in extremes of age (children less than 15 years old and adults older than 55 years old) and in people reporting higher exposure and closer contact to viral habitats such as swamps [8].
There is concern of not only natural outbreaks of EEEV due to the high morbidity and mortality that it causes, but also of the possibility of intentional outbreaks where EEEV could be used as a bioterror agent [5]. With EEEV, as with other Alphaviruses VEE and WEE, there is the potential for transmission not only by mosquito but also by aerosol. VEE, which has a lower infectious dose, has been reported as being studied in the US bioterror program in the 1960’s as an incapacitating weapon, but later had its stocks destroyed [16]. Characteristics such as the ability to be easily cultured in large amounts, long-term stability, possibility to be aerosolized, high rates of morbidity and mortality, no specific treatment, and lack of a licensed human vaccine, all make EEEV a bioterror candidate [16]. Therefore, EEEV is considered a select agent and figures in both NIAID (as a Category C agent) and CDC (as a Category B agent) lists [16]. In addition to natural outbreaks and the possibility of intentional outbreaks, four cases due to occupational exposure, in laboratorians working with EEEV, have been reported [16].

In South America infection in humans has been reported rarely, but presents with a similar pattern of North America in horses [4, 7, 8]. There are only two reported cases of EEEV encephalitis - one fatal case from Brazil in a 2 year old, and another case from Trinidad [6]. Even though cases of encephalitis due to EEEV are rare in South America, a study in Peru looking at febrile patients and healthy people in the Iquitos area, and testing for different Alphaviruses, found that 9/359 healthy volunteers and 3/153 febrile subjects had positive antibodies for EEEV. Febrile patients with EEEV antibodies had chills, arthralgia, muscle pain, headache, nausea, and vomiting but no signs of neurological manifestations [6].
There are differences in the ecology and epidemiology of EEEV virus between North America and South America including some already mentioned above like the most likely mosquito vector (*Cs melanura* vs. *Culex*), the possible reservoirs (birds vs. birds and/or small mammals), the length of the transmission season (early Summer to late Fall vs. almost year round), different strains of EEEV circulating (North American vs. South American strain), and the number of reported human infection (high vs. very low). In addition, Southern US states like Alabama and Florida share EEEV ecology features found in both North and South America [18-21].

**Blood Meal Analysis**

Blood meal analysis is an important tool to gather information on patterns of host preference which reflect the relationships among possible vectors and hosts in areas of disease transmission. It helps to understand more about the behavior of vector species possibly involved in arboviral transmission.

Early studies on host preference by mosquito vectors relied on methods in two basic categories: non-serological, which consisted of visual observation of bait mosquito traps, and serological methods [22]. Serological methods included variations of the precipitin tests such as “ring” test, microhematocrit tube, agar gel diffusion, microplate, and gel surface, and other tests such as the not widely used fluorescent antibody technique (FA), passive hemagglutination inhibition technique (HI), and enzyme-linked immunoabsorbent assay (ELISA) [22]. Precipitin tests required large amounts of plasma and lack the specificity and sensitivity of the tests available today while the other serological techniques listed above are around 100 times more sensitive than the
precipitin assay [22]. HI has been used widely but requires reagents that are time-consuming to produce (antiserum produced in rabbits or chicken and antibody coated red blood cells). The assay involves incubating the produced antiserum and the plasma from the blood meal, and then adding red blood cells coated with specific antibodies as the assay indicator. If the blood meal combines with the antisera, there is no subsequent agglutination of red blood cells and the reaction is positive (inhibition of hemagglutination). Another method is the latex agglutination test where latex beads are used as carriers in the reaction. However, latex agglutination is less sensitive than the precipitin test [22]. ELISA is a more sensitive technique that can be quantitative and automated [22].

More recently, molecular assays were developed for blood meal identification. These assays consisted of a great advance in the area of blood meal identification since this polymerase chain reaction (PCR) based assay is more sensitive and specific, more easily standardized, less prone to cross-reactivity, and less time-consuming than the old serological assays. Boakye et al, developed a (PCR)- heteroduplex analysis (HDA) assay. This assay is based on the amplification of the cytochrome B gene [23, 24]. Cytochrome B gene is used because it tends to be conserved within species but also tends to express a degree of variation among species [13]. Later, Lee et al, introduced a nested step into the protocol which made blood meal identification to the species level possible [24]. This assay has three steps. Initially DNA is isolated from the mosquito blood meal and used as a template in a PCR reaction that has conditions that favor amplification of cytochrome B gene from the blood meal and not from the mosquito. Later, the PCR product is annealed with a known amplified sequence of cytochrome B from a related avian species and the
differences in the two annealed sequences are separated by electrophoresis in the third step of the process [13]. Alternatively the identity of the blood meals can be performed through DNA sequencing of the PCR products, instead of observing the heteroduplexes and homoduplexes patterns of the HDA products on the gel.

Antibody against EEEV has been detected in many mammal and bird species [25]. In addition, many animal species have been reported as presenting with EEEV clinical disease including horses, pigs, dogs, cattle, camelids, deer, penguins, and seals. Many factors seem to influence patterns of preferential feeding by disease vectors including host preference, host abundance, and host defenses [22]. Moreover, vector host preference seems to differ not only across different host classes but also within a host class (among different species of the same class), and depending on the specific site where viral transmission occurs [15, 26, 27].

Advances in blood meal analysis techniques have made it possible to observe temporal shifts to or away from certain host classes for arbovirus vectors, with avian hosts being targeted early in the season, followed by a shift to mammalian hosts later in the year [15, 26-29]. Using this technique we looked at host preference profiles in an area of EEEV transmission in Alabama.

Research Design and Methods

**Overall Study Design**

In order to look at the host preference profile in a area of EEEV transmission in Alabama, we conducted a secondary data analysis of a serial ecological survey of mosquito vectors and bird host species involved in EEEV transmission at the Tuskegee
National Forest (TNF) site (Auburn, Alabama). Data has been collected from 2001-2004, and 2006-2007.

**Study Site**

The study site is located within the Tuskegee National Forest, located 17 miles southwest of Auburn, in Macon County, Alabama (32°00’26_W, 85°44’38_N). This site was chosen by virtue of its proximity to a number of previously reported equine EEEV cases and has been previously described [20, 30]. In summary, it is an area of around 10-15-acres, 3 kilometers of the town square of Tuskegee where there has been extensive reforestation over former farmland that was abandoned in the early 1900s. The area has rich vegetation and the water from five beaver ponds in the area provide most of the standing water in the area.

**Mosquito Collection**

Mosquitoes were collected from the study site using multiple methods including CDC light traps, vegetation sweeps and vacuum collections of resting boxes and natural resting sites. The use of multiple collection methods allowed for collection of a more broad number and variety of mosquito species and also different mosquitoes of the same species (like male/female, nulliparous/parous female mosquitoes). Blood engorged or gravid mosquitoes are usually collected in resting boxes, and vacuum collection aids in collection of species not attracted to light. Collections began the first week of May and continued through the end of September, and collections from each box/site were made twice a week on the same day of each week and at approximately the same time.
(8:30–10:30 AM) each day. Light traps run from dusk to dawn and were positioned approximately two meters above ground. Vacuum collections complemented those from light traps. Collections were carried out in the wooded interior of the site and the material was returned to the laboratory, sorted, and identified using a chill table and binocular microscope, and blood-fed individuals were frozen at −70°C [20, 30].

**Blood Meal Analysis Assay**

This technique has been previously published [13, 24, 30, 31]. First-stage PCR amplifications was conducted in a solution containing 60 mM Tris-HCl (pH 8.5), 15 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM of each primer, 1.25 units of Taq DNA polymerase (Roche Biochemicals, Indianapolis, IN), and 2.5 µL of DNA template (total genomic DNA from mosquito blood meal). The sequence of the primers in the PCR are: 5’CCCCTCAGAATGATATTGTCTCTCA3’ and 5’CCATCCAACATCTCAGCATGATGAAA3’. Reactions began with an incubation at 95°C for 3.5 minutes, followed by 40 cycles at 95°C for 30 seconds, 60°C for 50 seconds, and at 72°C for 40 seconds. The reaction was completed by incubation at 72°C for five minutes. Nested amplifications were carried out in a 50-µL volume containing 60 mM Tris-HCl (pH 9.5), 15 mM (NH₄)₂SO₄, 2.0 mM MgCl₂, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM of each primer, 1.25 units of Taq DNA polymerase (Roche Biochemicals), and 0.5 µL of the first step amplification product. The sequence of the primers used in the nested PCR were 5’-TCWRCHTGATGAAAACTTCGG-3’ and 5’- GTTGCYATKAGGGYYAGGAG-3’ where W = A or T, R = A or G, H = A, C or T, Y = C or T, and K = G or T. Amplification conditions were 95°C for three minutes,
followed by 40 cycles at 95°C for 30 seconds, 55°C for one minute, and 72°C for one
minute. The reaction was completed with a seven minute extension at 72°C. Two aliquots
of 6 µL of the PCR product were then separately mixed with 6 µL of PCR product driver
derived from northern cardinal (*Cardinalis cardinalis*) and Carolina chickadee (*Poecile
carolinensis*). The combined sample and driver PCR products were mixed with 8 µL of
10 mM Tris-HCl (pH 8.0), 1 mM EDTA and overlaid with 10 µL of mineral oil. The
mixture was denatured at 99°C for 2.5 minutes and allowed to form heteroduplex
products by slow cooling to room temperature. The identity of the blood meals can be
done either by running HDA products on a polyacrylamide/urea gel and observing the
patterns of the homoduplex and heteroduplex products or by doing DNA sequence
analysis of the PCR products. The identification by electrophoresis was done as follows:
an aliquot (14 µL) of each heteroduplex solution was mixed with 6 µL of loading buffer
(0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 108 mM Tris-boric acid,
pH 8.0, and 2.7 mM EDTA). Ten microliters of this mixture were loaded onto a 5%
polyacrylamide/urea gel (29:1 acrylamide:bisacrylamide, 1 M urea) prepared in 108 mM
Tris-boric acid (pH 8.0), 2.7 mM EDTA. Electrophoresis was conducted on 20 cm × 20
cm Protean II Xii system (Bio-Rad, Hercules, CA) at 12 mA per gel for 18 hours in 89
mM Trisboric acid (pH 8.3), 2.5 mM EDTA. Gels were stained in Sybr green (Molecular
Probes, Eugene, OR) and homoduplex and heteroduplex patterns were visualized under
ultraviolet light. Samples were grouped based upon their HDA product mobilities in the
northern cardinal and Carolina chickadee HDA assays.
Statistical Analysis

The number of mosquitoes from each species present in the study area was calculated for each year at each collection date during a transmission season (May to September) for the years of 2001-2004, and 2006-2007. The number and identity of blood meals from each mosquito species at each time point were calculated based on the HDA analysis and DNA sequence. Blood meals were then classified into host classes: avian, mammal, amphibian, or reptile. Analysis was also done using normalized blood meal counts. Normalized variables were obtained by dividing blood meal counts by the number of collection days for the period, by the total number of blood meals analyzed during a given time period, by the number of blood meals taken from the same class as the host species (avian or mammal) for a given time period, and by the number of blood meals of the same host species taken over the course of the year.

We used two statistical tests in our analysis: Runs test, which is a test of randomness, and Two-way Analysis of Variance – Tukey’s 1 degree-of-freedom for non-additivity, which is a test of interaction. Runs test [32], is a non-parametric test (i.e. one which does not assume the normal or any other particular distribution), and is commonly used to answer the question “Was this string of values generated from a random process?”. The null hypothesis is random order. In our specific case, if the number of blood meals over time for a particular host species was tested using Runs test and the p-value associated with that analysis was >0.05, it would lead us to fail to reject the null hypothesis that the frequency of blood meals over time was generated randomly.

Interaction between two variables occurs when values of one variable are not consistently distributed across different levels of the other variable, and is usually tested
when you have more than one observation per cell (or repetition) by using the two-way ANOVA method [33]. However, Tukey [34], in 1949, developed a test to assess interaction in a two-way model with one observation per cell (no repetition). In the current study, given that a number sequence for blood meal counts by a specific host is not random, we used the Tukey’s one degree-of-freedom for non-additivity to test for interaction. A non-significant p-value (>0.05) puts us in a position of failing to reject the null hypothesis of no interaction, or in other words, a significant p-value suggests that the pattern of feeding upon a specific host at the TNF site is not consistent from year to year.

**Review Paper**

This dissertation also contains a review paper on the differences and similarities of the epidemiology of EEEV in North and South America. References in this review article were found by doing a literature search in Pubmed using a variety of words including: EEE and its variations (eastern equine encephalitis, EEEV, EEE virus), epidemiology, ecology, and names of countries in South and Central America. In addition, all references in each publication found by this method were reviewed for additional references related to the topic of the paper. It includes publications in Portuguese and in Spanish as well as manuscripts in English published in Brazilian journals that are less familiar and less accessible for audiences outside the country of publication.
HOST PREFERENCE PROFILE IN AN AREA OF EASTERN EQUINE ENCEPHALITIS (EEE) VIRUS TRANSMISSION IN ALABAMA.

by

ANA L. OLIVEIRA, SIBYLLE KRISTENSEN, CHARLES KATHOLI, HASSAN K. HASSAN, NATHAN D. BURKETT-CADENA, AND THOMAS R. UNNASCH.

Submitted to the Journal of Vector Borne and Zoonotic Diseases

Format adapted for dissertation
ABSTRACT

Temporal shifts among host classes in feeding patterns of the mosquito vectors for arboviruses are believed to be an important factor in the ecology of these viruses. The significance of temporal changes in the host preference profile of mosquito vectors involved in Eastern Equine Encephalitis Virus (EEEV) transmission at a site at the Tuskegee National Forest (TNF), Alabama, has been investigated employing a dataset containing a total of 1,583 identified blood meals from 90 different host species collected over six years. Runs test (a test of randomness) revealed that blood meals from the Great Blue Heron (GBH) and White-tailed Deer (WTD) (two of the most fed upon species) were not random across years; while meals derived from the Yellow-crowned Night Heron (another of the most frequently fed upon species) appeared to be randomly distributed. Tukey’s two-way ANOVA with 1 degree-of-freedom (test for interaction) showed that normalized blood meal counts for WTD and GBH were consistent from year to year. These data suggest that the temporal profile of feeding shifts for certain species exhibited a non-random pattern that was consistent from year to year, suggesting an underlying biological mechanism for the observed feeding patterns, in which some species are preferentially fed upon while other species are more opportunistically targeted.
INTRODUCTION

Eastern Equine Encephalitis virus (EEEV) usually circulates in a mosquito-avian (enzootic) cycle within well-defined habitats, but outbreaks in humans and horses can also occur (epizootic transmission) [2, 7, 9, 10, 15]. The ecology of EEEV, similar to other arboviruses, involves many variables in a complex and dynamic system. In order to better understand factors that trigger epizootic transmission of EEEV, and to help prevent and control these outbreaks, it is necessary to better understand key ecological factors in EEEV transmission, such as host preference behavior by EEEV mosquito vectors [15, 30].

Many animal species have been reported as presenting with EEEV clinical disease including horses, pigs, dogs, cattle, camelids, deer, penguins, and seals. In addition, antibody against EEEV has been detected in many mammal and bird species [25]. Many factors seem to influence patterns of preferential feeding by disease vectors including host preference, host abundance, and host defenses [22]. In addition, vector host preference seems to differ not only across different host classes but also within a host class (among different species of the same class), and depending on the specific site where viral transmission occurs [15, 26, 27].

Studies of the host preference of arbovirus vectors have been facilitated by the development of PCR-based assays capable of identifying the source of a blood meal to the species level [28, 29]. Using these methods, several authors have observed temporal
shifts to or away from certain host classes for arbovirus vectors, with avian hosts being targeted early in the season, followed by a shift to mammalian hosts later in the year [15, 26, 27]. The objective of the present study was to investigate the significance of such shifts in the host preference profile of mosquito vectors involved in EEEV transmission at the Tuskegee National Forest (TNF), Alabama, and to determine if these shifts were consistent between transmission seasons by using known statistical tests. If this was the case, such shifts in feeding upon particular host species might be indicative of an underlying biological mechanism that could be an important driver in the dynamics of arbovirus transmission.

MATERIALS AND METHODS

Study Site

The study was carried out within Tuskegee National Forest (TNF), 17 miles southwest of Auburn, in Macon County, Alabama (32°00’26 W, 85°44’38 N). This site was chosen by virtue of its proximity to a number of previously reported equine EEEV cases and has been previously described in detail [20, 30].

Mosquito Collections

Mosquitoes were collected from the study site using a combination of CDC light traps, vegetation sweeps, and vacuum collections from artificial resting shelters and natural resting sites beginning the first week of May and continuing through the end of September. Collections from each box/site were made twice a week at approximately the same time (8:30–10:30 AM) each day. Samples were returned to the laboratory, sorted,
and identified to species. Blood-fed mosquitoes were frozen at −80°C until blood meal identification [20, 30].

Blood Meal Analysis

The identity of host blood meals at the study site was determined using a vertebrate specific PCR as previously described [15, 30, 31, 35].

Statistical Analysis

*Runs Test*

Runs test [32], a non-parametric test (i.e. one which does not assume the normal or any other particular distribution), is commonly used to answer the question “was this string of values generated from a random process?” This test calculates the mean of all observations and then the deviations from the mean. If the deviation is positive, it is scored as a 1 and if negative, as a minus 1. The result is a string of positive and negative values. Runs test counts the number of transitions from positive to negative, and negative to positive. The exact distribution of this test statistic is easily calculated under the null hypothesis of random noise. Therefore, a non-significant p-value (>0.05) leads to failure to reject the assumption of random order.

*Two Way Analysis of Variance – Tukey’s 1 degree-of-freedom for non-additivity (testing interaction)*

Interaction between two variables occurs when values of one variable are not consistently distributed across different levels of the other variable. Interaction is usually
tested when you have more than one observation per cell (or repetition) by using the two-way ANOVA method [33]. However, Tukey [34], in 1949, developed a test to assess interaction in a two-way model with one observation per cell (no repetition).

In our particular case, given that a number sequence for blood meal counts by a specific host is not random, we used the Tukey’s one degree-of-freedom for non-additivity to test for interaction. A non-significant p-value (>0.05) puts us in a position of failing to reject the null hypothesis of no interaction, or in other words, a significant p-value suggests that the pattern of feeding upon a specific host at the TNF site is not consistent from year to year.

RESULTS

A total of 1,583 blood meals were identified from all years of the study (2001-2004, 2006-2007) from 90 different host species (Table 1). *Culex erraticus* composed the great majority of mosquitoes used in the study, accounting for 65.6% to 94.4% (Table 2) of identified blood meals for all five top host species.

Runs test was initially used to test the hypothesis that feeding upon the different host species was not randomly distributed across time. To accomplish this, host meals were grouped into 10 semi-monthly time categories according to the date they were collected (May to September). The analysis was restricted to blood meals obtained from White-tailed Deer, Great Blue Heron, Yellow-crowned Night Heron and Northern Cardinal, as these were the most commonly fed upon species for which blood meals were identified on all years of the study. Results for White-tailed Deer and Great Blue Heron showed a statistically significant value (p < 0.001), so we rejected the hypothesis that
blood meal counts for these two host species were randomly distributed over time. However, p-values for the Runs test for Yellow-crowned Night Heron and Northern Cardinal were 0.147 and 0.113 respectively, indicating in this case that blood meal counts for these hosts were likely to have been randomly distributed over time.

It was possible that the Runs test results were confounded by variations in the number of blooded mosquitoes collected. For this reason, we normalized blood meal counts by dividing them by the number of collection days for the period, by the total number of blood meals analyzed during a given time period, by the number of blood meals taken from the same class as the host species (avian or mammal) for a given time period, and by the number of blood meals of the same host species (Great Blue Heron or White-tailed Deer) taken over the course of the year. These new normalized variables for both Great Blue Heron and White-tailed Deer were tested with Runs and Tukey’s 1 degree-of-freedom tests. For Great Blue Heron and White-tailed Deer the raw blood meal count data analyzed using this method resulted in p-values of <0.001 indicating no consistency in the temporal feeding pattern across years. However, when normalized by the total number of avian (for Great Blue Heron blood meals) and mammalian (for White-tailed Deer) blood meals for the each time period both showed a non-significant p-values (0.337 for Great Blue Heron and 0.187 for White-tailed Deer), indicating that the temporal pattern of the proportion of avian blood meals taken from Great Blue Heron, and the proportion of mammalian blood meals taken from the White-tailed Deer were consistent across the studied years. Similarly when the number of meals taken from White-tailed Deer for each time period were normalized for the total number of meals taken from this species over the course of the entire year, a p-value of 0.067 was
obtained, also suggesting that the proportion of the annual White-tailed Deer blood meal taken during each semi-monthly period was consistent from year to year (Table 3).

Next, similar analyses were conducted on blood meals derived from *Cx. erraticus*, again limiting the analysis to the five most commonly fed upon hosts. *Culex erraticus* is the most abundant mosquito species at the site, and has been implicated as a potential vector for EEEV in the Southeastern USA [20, 36]. Based on the Runs test, blood meals from *Cx. erraticus* identified as coming from Green Heron and White-tailed Deer were not randomly distributed (Table 4). Similarly, *Cx. erraticus* blood meals from the Green Heron and Northern Cardinal, when normalized by host class for each time period, showed marginally statistically significantly p-values on the test of randomness (Runs test; p-value = 0.07 and 0.08 respectively; Table 4) what leads us to not totally reject the possibility of a pattern. Tukey’s test results indicated that *Cx. erraticus* blood meals derived from the Green Heron, Northern Cardinal and White-tailed Deer, when normalized by host class seemed to be consistent from year to year (p>0.05; Table 4).

**DISCUSSION**

The Runs test results suggest that blood meal counts for Great Blue Heron and White-tailed Deer were not generated from a random process. This is in accordance with the observed temporal peaks in blood meal counts for these two hosts. Great Blue Heron blood meal counts were highest from late May to late July while White-tailed Deer were highest from late June to early September. Since Great Blue Heron and White-tailed Deer were the most common avian and mammalian hosts respectively, this temporal characteristic seems to reflect a host preference shift from avian to mammalian hosts.
This host shift has been reported for vectors of another arbovirus, West Nile virus, where robins are targeted early in the season, and humans become the main host later in the transmission season [13, 27].

Even though the raw blood meal counts for the Great Blue Heron and White-tailed Deer showed no consistency over the years when queried using Tukey’s 1-way test, the normalized data for those species did follow a similar pattern year to year when normalized for the total number of meals from their respective classes (host blood meal counts divided by the total number of same class blood meals for the same period). The Great Blue Heron has been previously reported as a preferred host species for potential EEEV vectors [21, 30]. However, this possible preference by EEEV vectors has been largely attributed to this avian species size and less to actual preference per se, since Great Blue Heron feeding index was less dramatic when normalized to its biomass [21, 30]. In addition to body size, behavior of these birds, such as their ambush predatory strategy and their high level of tolerance to mosquito bites have been cited as plausible explanations for the high frequency of Heron blood meals in areas of arbovirus transmission [37].

Given the high rate of feeding of mosquitoes upon the White-tailed Deer, it seems plausible that deer might be frequently exposed to EEEV in areas where this virus is circulating. Little has been reported on seroprevalence of EEEV, or other arboviruses for that matter, in White-tailed Deer. Older reports on the seroprevalence of arboviruses in wild mammals found White-tailed Deer seropositive for many arboviruses [38, 39] including EEEV [40]. However, more recent studies have reported not only White-tailed Deer as a source of blood meals from Culiseta melanura [41], but also with confirmed
EEEV infection, high seroprevalence for EEEV, and clinical EEEV disease [42, 43]. These findings provide evidence that deer are frequently exposed to, and may become infected with EEEV. However, the role, if any, that deer play in the transmission dynamics of EEEV is yet to be determined.

The consistent temporal pattern of feeding upon some species, such as the Great Blue Heron and White-tailed Deer, suggests that this pattern might be driven by an underlying biological process. This process might relate to changes in the biology of the mosquito species over the season, or might relate to changes in the life history or behavior of the hosts in question. Further studies exploring the changes in the life history or behavior of these species in relation to the mosquito feeding upon these species will be necessary to uncover the potential biological basis for this temporal pattern.

ACKNOWLEDGEMENTS

This research was supported by a grant from the National Institute of Allergy and Infectious Diseases, Project # R01AI049724 to TRU. Numerous field assistants helped with mosquito collections, including N. Click, J. Camp, X. Yue, C. Cazalet and K. Gray. The authors thank Drs. Robert Novak and Craig Wilson, from the University of Alabama at Birmingham, for critically reading this manuscript.
REFERENCES


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*includes 86 species: 11 mammalian, 44 avian and 15 amphibian, and 16 reptile.

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Table 3. Runs Test (Test of Randomness) and Tukey 1-way Test (Test for Interaction) on Host Blood Meal Counts at Tuskegee National Forest (2001-2004, 2006-2007).

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<td>GBH blood meal counts/ total GBH blood meal counts for the same year</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GBH blood meal counts/ total blood meals for the same year</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Runs Test (Test of Randomness) and Tukey 1-way Test (Test for Interaction) on Host Blood Meal Counts from *Culex erraticus*, Tuskegee National Forest (2001-2004, 2006-2007).

<table>
<thead>
<tr>
<th>Host Class Blood Meal Counts</th>
<th>p-value on Runs Test</th>
<th>p-value on Tukey’s Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Blue Heron (GBH)</td>
<td>0.238</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Green Heron (GH)</td>
<td>0.012</td>
<td>0.137</td>
</tr>
<tr>
<td>Northern Cardinal (NC)</td>
<td>0.078</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>White-tailed Deer (WTD)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Yellow-crowned Night Heron (YCNH)</td>
<td>0.786</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood Meal Counts/Host Class Blood Meal</th>
<th>p-value on Runs Test</th>
<th>p-value on Tukey’s Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Great Blue Heron (GBH)</td>
<td>0.106</td>
<td>0.475</td>
</tr>
<tr>
<td>Green Heron (GH)</td>
<td>0.070</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Northern Cardinal (NC)</td>
<td>0.078</td>
<td>0.264</td>
</tr>
<tr>
<td><em>White-tailed Deer (WTD)</em></td>
<td>0.014</td>
<td>0.932</td>
</tr>
<tr>
<td>Yellow-crowned Night Heron (YCNH)</td>
<td>0.205</td>
<td>0.619</td>
</tr>
</tbody>
</table>
EPIDEMIOLOGICAL DIFFERENCES OF EASTERN EQUINE ENCEPHALITIS VIRUS (EEEV) IN THE AMERICAS.

by

ANA L OLIVEIRA, ROBERT NOVAK, CRAIG WILSON, AND SIBYLLE KRISTENSEN

In preparation for Revista Panamericana de Salud Publica/Pan American Journal of Public Health

Format adapted for dissertation
ABSTRACT

Objective. The objective of the current manuscript is to review the epidemiology of EEEV focusing on the differences between North and South America. Methods. A systematic review of the literature was performed in order to identify publications that exemplified and/or discussed the differences in the epidemiology of EEEV between North and South America. Results. 60 citations were included in this manuscript, including 10 published in Portuguese. Eastern Equine Encephalitis virus (EEEV) is present throughout the Americas and the Caribbean, although some geographical differences in the ecology and epidemiology of the virus exist. The North American type of EEEV is associated with severe disease in both humans and horses. EEEV is transmitted by the enzootic vector Culiseta melanura among birds in northern North America, while Culex melanoconion has been implicated as the enzootic vector and small mammals and birds may serve as EEEV hosts in South America. The South American EEEV strain in horses can present similarly to the North American type. However, in humans, infection is rare and usually presents with asymptomatic or milder form of the disease. Possible explanations for these epidemiological differences relate to distinct surveillance approaches, ecological discrepancies, and biological disparities between the EEEV that circulates in North and South America.
INTRODUCTION

Eastern Equine Encephalitis virus (EEEV) is a positive single stranded RNA Alphavirus (1-3). Its transmission cycle includes a vertebrate host, and an arthropod vector, involved in the transmission of the virus. There are two types of hosts: common hosts like birds and small mammals, and the so called “dead-end hosts”. In dead-end hosts, such as humans and horses, the virus does not reach high levels in the circulation and thus cannot infect an arthropod vector, breaking the cycle of infection (4, 5). EEEV normally circulates in a mosquito-avian cycle (enzootic) usually within focal sites in and around coastal and inland swamps, but outbreaks in birds, horses, and humans can also occur (epizootic/epidemic transmission) (4, 5).

EEEV is present throughout the Americas and the Caribbean, although some differences in the ecology and epidemiology of the virus between North and South America have been reported (2, 4, 6, 7). EEEV infection often presents with high mortality and severe morbidity. Encephalitis due to EEEV infection most often results in costly long term health care (8). Other characteristics of EEEV, such as the possibility of being aerosolized (and remain infectious in the aerosol form), being easy to culture and highly stable, the lack of both specific treatment and a licensed human vaccine make EEEV (and other Alphaviruses) figure in both the National Institute of Allergy and Infectious Diseases (NIAID), as a category B agent, and the Centers for Disease Control and Prevention (CDC), as a category C agent, lists of selective agents (1, 9, 10). The
objective of this work is to review the epidemiology of EEEV focusing on the main differences between North and South America.

EEE virus

EEE is a positive sense single stranded RNA enveloped virus of about 11-12kb in length (2, 5, 6). EEEV has three structural proteins: capsid (C), envelope glycoprotein 1 (E1), and envelope glycoprotein 2 (E2). EEE viral genome also encodes for four non-structural proteins (nsP1, nsP2, nsP3, and nsP4) (2, 3, 6, 7, 11). In addition, EEE is an Alphavirus of the family Togoviridae and the only member of the EEE antigenic complex.

Clinically, infection due to EEEV manifests with a range of symptoms from subclinical febrile illness to severe encephalitis (1, 12). Initially EEEV infection has a systemic presentation where the virus replicates in peripheral sites. Symptoms of the systemic phase of infection include fever, chills, malaise, and myalgia. These systemic symptoms usually last 1-2 weeks and can either resolve or progress to encephalitis (symptoms include fever, headache, vomiting, seizure and coma) (9, 12). Encephalitis can have an abrupt onset or appear after a few days of systemic disease, and death can occur after 2-10 days of onset of symptoms (9, 12). The death rate in horses can reach 90% with 66% of survivors presenting with sequelae. In humans, mortality rate can reach 30%-50% with half of survivors presenting with neurological sequelae (9). It has been reported that case fatality rate for EEEV infection in children can be as high as 70% and four laboratory infections have been associated with EEEV (9). Neurological sequelae can vary in severity but are usually associated with increased mortality rates, estimated to
be around 90% at nine years post-infection with only a 3% survival rate (12). Little has been published concerning the cost of disease caused by EEEV, but a calculation expressed in 1990 dollars estimated the lifetime cost of a single case can be close to 3 million dollars (8). There is no specific treatment for EEEV infection (7). However studies in mice have investigated chemical compounds and their ability to inhibit disease caused by Togoviridae family viruses VEE and Semliki Forest including recombinant interferon (9). There is an experimental EEEV vaccine for humans recommended for laboratory technicians working with the virus, and a licensed vaccine for horses (9, 12).

There are 2 antigenic types of EEEV which can be differentiated based on kinetic hemagglutination-inhibition reactions. The North American type of EEEV occurs in parts of USA, Canada, and the Caribbean while the South American type occurs in Central and South America (4). The North American type is associated with severe disease in both humans and horses presenting with high mortality and permanent neurological sequelae in survivors. The South American type in horses can present similarly to the North American type with high mortality and morbidity. However, in humans, infection is rare and usually presents with asymptomatic or milder form of the disease (2, 4, 6). The South American type has a higher degree of heterogeneity (2, 6). Other evidence of antigenic distinction between the North American and the South American types of EEEV is that humans vaccinated with the North American strain vaccine do not develop neutralizing antibodies to the South American strains (4, 6). There are 4 EEEV subtypes based on genetic information: lineage I, II, III, and IV. Lineage I circulates in North America, while II, III, and IV circulate in South America (2, 4, 6, 13). The 4 major EEEV lineages differ by 20-29% at the nucleotide sequence level and by 5-10% in structural protein
amino acids (4). Lineage I contains isolates from Canada, USA and the Caribbean. Lineage II is represented by viruses from Brazil, Peru and Guatemala. Lineage III has isolates from Argentina, Guyana, Ecuador, Panama, Trinidad, and Venezuela while lineage IV is represented by a single isolate from Brazil (4, 6).

EEEV IN NORTH AMERICA

In North America, EEEV can be found from southeastern Canada to southeastern USA in focal sites around swamp areas (6, 14, 15). EEEV is the causative agent of severe acute encephalitis in humans and horses with human EEEV cases in North America occurring sporadically with an average of 5 cases/year (16). A serological study in New Jersey, during an outbreak of EEEV in 1959 estimated that the mean ratio of symptomatic to asymptomatic infections for EEEV was 1:23 (ranging from 1:16 to 1:23) (12, 16). This ratio of symptomatic/asymptomatic infections may be higher in adults (1:40) (12). Reimann et al, 2008, analyzing data reported to the CDC National Notifiable Disease Surveillance System (NNDSS) passive surveillance system (which tends to underestimate the true number of cases) by state health departments, calculated that the national cumulative incidence of EEEV neuroinvasive disease in the US was 0.002 per 100,000 people per year for 1990-1998 (pre-West Nile introduction in the US) and 0.003 per 100,000 people per year for 1999-2007 (post-West Nile). There was no statistically significant difference in annual incidence of EEEV neuroinvasive disease by age or by race (African American vs. White). However, males presented an incidence two times higher than the incidence of EEEV neuroinvasive disease in females (17).
The North American EEEV type is mostly transmitted by the enzootic vector *Culiseta melanura*, which is a very ornitophilic mosquito species, among passerine birds in coastal and inland swamp areas in northern North America (4, 6, 7). All the factors that initiate epizootic transmission are not well understood, but it is believed that in situations when the vector population reaches higher levels, epizootic transmission of EEEV can occur affecting humans and other animals through the participation of other mosquito species with less restricted feeding behavior than *Cs. melanura*. In temperate climates such as North America, transmission is seasonal and peaks in late summer (August) and early fall (September) (4, 6, 14).

Many hypotheses have been suggested to explain the maintenance of EEEV in North America. One of these hypotheses is that EEEV would be re-introduced every year in a transmission area by migrating birds. However, most published observations do not support the role of migrating birds in the maintenance of EEEV in North America. For example, studies in New Jersey found young birds virus positive before mosquitoes could be found positive indicating that factors other than migratory birds are responsible for the maintenance of EEEV in that area (4, 18). In addition, resident birds as opposed to migratory birds are usually identified as enzootic vectors, suggesting that activation of latent infection in resident birds could be responsible for the maintenance of the virus in temperate climates (4). Another proposed hypothesis is the over-wintering of the virus by means of transovarial transmission. However, that hypothesis has also been discounted by laboratory and field data showing the inability to reproduce transovarian transmission in the lab, and the lack of isolation of infected male mosquitoes or infected larvae (4, 6).
EEEV may be capable of over-wintering in its endemic foci in other animals such as birds, amphibians or reptiles (19).

Passerine birds have been reported as the most likely vertebrate hosts responsible for maintaining EEEV in North America. However, studies in Southern US, recently suggested that mosquito vectors for EEEV (and WNV) feed upon certain bird species, including non-passerine species, such as herons, at rates that are above what would be predicted based upon their abundance (20-24). These studies further suggest that mosquito vectors may preferentially feed upon the young-of-the-year of particular bird species. Nestlings have been suggested to be important vertebrate hosts for EEEV amplification (25) because birds in this age group are more susceptible to infection than adults, develop viremia of greater magnitude and duration, and are less defensive toward host-seeking mosquitoes (25, 26).

Research done in Florida and Alabama has suggested that the epidemiology of EEEV in the southeastern USA might be more similar to the epidemiology of EEEV in South America (19, 20, 26, 27). Southeastern US states like Florida and Alabama exhibit characteristics that are intermediate to North and South America. In both states, Florida and Alabama, the EEV circulating is one associated with higher rate of human cases, the North American type. In these states, climate is more similar to South America and although EEEV is thought to circulate most of the year (like in South America), peaks of viral transmission are still observed (like in North America) (26). However, the mosquito vector implicated with EEEV transmission is not *Cs melanura* (although present in those states), as in Northern North America, but *Culex* mosquitoes more related to *Cx melanocoonion*, the reported possible EEEV mosquito vector in South America (19, 27).
EEEV IN SOUTH AMERICA

In South America, due to the very low number of reported EEE human infections, the epidemiology of EEEV has been less studied (4). It is believed that in more tropical climates such as South America, the Caribbean, and Florida EEEV transmission occurs more continuously as opposed to the seasonal pattern found in temperate climates such as in Northern North America (4, 6). *Culex* mosquitoes subgenus *melanoconion* has been implicated as the enzootic vector and small mammals (such as rodents and marsupials) and/or birds serve as enzootic hosts for EEEV in South America (4, 6, 7, 11, 28-33).

Epidemics in horses occur in South America with morbidity and mortality comparable to North America’s, and according to data from studies in horses, humans, other mammals, birds, and mosquitoes, there is indication that many arboviruses circulate in South America, including EEEV (13, 30, 33-41).

In Brazil, three percent (16/556) of avian samples tested were sero-positive for EEEV from forest birds with black vulture showing higher antibody rates, and one viral isolation from *Columbigallina passerine* (dove) (29). Other avian species have been also found seropositive for EEEV: Ochre-bellied Flycatcher, Silver-beaked Tanager, Black-spotted Bare-eye, White-shouldered Antshrike (highest percent positive at 4%), Fasciated Antshrike, and Yellow-rumped Cacique (30), Ash-throated Crake, Ruddy Ground Dove, Guira Cuckoo, Spix’s Spinetail, White-browned Foliage Gleaner, Cattle Tyrant, House Wren, House sparrow, sayaca tanager, masked yellow-throat, palm tanager, double-collared seedeater, ruby-crowned tanager, and rufous-collared sparrow (36), Red-eyed Vireo, White-necked Thrush, Trush (*Turdus ruficeps*), Rufous-capped Antthrush, Ruby-
crowned Tanager, *Phylidor atricapillus*, Planalto Tyrannulet, *Elaenia sp* (42), and wild birds from Mexico and Guatemala (40).

EEEV has been also isolated from rodents (including *Oryzomys spp*) and opossum (for example *Didelphis marsupialis*) (42). Sero-positivity for EEEV has been observed in opossum (*Marmosa cinerea*) (29, 30, 32), amphibians and/or reptiles (30, 32), rodents (30), pig, dog, wild mammals (40). In addition, EEEV has been isolated from sentinel monkey, mice (29, 30, 43), chicken (29, 30), and hamsters (31, 32, 38, 44). By serological testing sentinel chicken and hamsters have been found EEEV positive (29, 41).

In the Amazon region of Brazil, for example, surveillance for arbovirus has been ongoing since the 1950’s and human seroprevalence up to 25% has been reported compared to an average of around 1% for Brazil as a whole (30). In that region, reported seroprevalence for EEEV in humans has ranged from 0.06%-25% (29, 30, 43, 45) and from 0%-27% in horses (29, 43, 46).

Vale do Ribeira and Baixada Santista areas of Sao Paulo state, Brazil, consist of an area of arbovirus circulation and has experienced epidemics including the in the late 70’s due to Rocio virus. In the post-epidemic period, when EEEV was included in the list of tested arboviruses, seroprevalence for EEEV was 25% (1/4) in the general population (47), 5.17% (29/561) in patients without symptoms at a local hospital (48), and 1.06% (2/189) in school children 6-14 years old (49). Serological survey in residents of an ecological reserve in the same area, found neutralizing antibodies to EEEV in 2.19% (4/182) of samples (35).
Arbovirus studies have also been conducted in other Brazilian states. For example, a sero-prevalence study in horses in Parana state, in 1997 sampled horses more than 2 years old, with without encephalitis symptoms, and no history of vaccination and 52.55% (12/22) sera were positive for EEE (50). In the Pantanal region of Brazil, a region equivalent to the swamp areas in the USA around the Great Lakes and in the Atlantic seacoast, neutralizing antibodies to EEEV have been detected by serological tests in 6.7% of 432 horses from 5 farms during 1992 (34).

Other countries in South America other than Brazil have also reported EEEV circulation without reports of clinical disease. For example, seroprevalence reports in humans in Venezuela ranged from 0% (37) to 2.08% (Yanomani Indians) (38). In Argentina there have been reports of seroprevalence for EEEV of 0.47% (1/213) in humans and 7.32% (3/41) in horses (39). In Panama, serology showed that 100/803 (12.45%) sera from horses were positive for EEEV (33), and 0.32% (4/1,255) human sera samples from Guatemala and Honduras have been reported EEEV positive samples (40). In Mexico, EEEV observed seropositivity in humans was 6.74% (12/178) (40), while research from Peru have showed seropositivity for EEEV ranging from 20-40% in horses (41) and 2.51% (9/359) in humans (10).

Despite this high reported seroprevalence, differently from what happens in North America, only two clinical cases of human EEEV infection have been reported in South America: one in Bahia state, in the Northeastern part of Brazil, in the 1950's (30) and another case from Trinidad. Confirmation of the Brazilian case was made through virus isolation in a two year old girl admitted to the local hospital on May 1955. She experienced flu like symptoms (cough, sneezing) and fifteen days later had high fever
accompanied by convulsions that progressed to coma and death which occurred after one
day of hospital admission. Brain autopsy revealed unspecific encephalitis (51).

DISCUSSION

Many hypotheses have been postulated to explain the observed distinctions in
reported human EEEV disease rates between North and South America. They relate to
the ecology of the virus, disparities in surveillance activities, and differences in the
biology of the virus that circulates in these two geographical areas. The much lower
number of reported human EEEV infections in South America compared to North
America has been attributed in part to less surveillance efforts or less sensitive
surveillance for this virus in South America (52). However, numerous surveys for EEEV
have been performed, looking for EEEV antibodies in sera, and virus in blood or tissues
of possible hosts, and for EEEV in possible vectors in South American countries such as
Brazil, Argentina, Trinidad, Venezuela and Peru. These surveys, even when found EEEV
positive antibodies or was able to isolate virus from those samples, failed to find reports
of humans presenting with clinical EEEV disease. In instances when EEEV was not
found in those areas, other human causing arboviruses were detected.

Symptoms of EEEV are very unspecific and can have the same clinical
presentation as other arboviruses circulating in the area. The list of differential diagnosis
for EEEV encompasses other arboviruses including WEE, VEE, SLE, LAC, Rocky
Mountain Spotted Fever, bacterial diseases like enterovirus, leptospirosis, bacterial
meningitis, vial diseases like herpes, mumps, influenza, adenovirus, AIDS encephalitis,
and other non-infectious diseases such as stroke, brain tumor, non-infectious CNS
diseases, Alzheimer’s, long term alcohol abuse, dementia, and acute rheumatic fever among others (12). In South America, a great number of arboviruses co-exist, so it is possible that some degree of misclassification based on clinically diagnosis alone or less specific laboratory assays (such as hemaglutination inhibition and neutralization assays) as of other arboviruses could actually be due to EEEV infection (12, 30, 48).

EEEV occurs in localized areas, and epizootics occur at certain time intervals. Human cases could be occurring but not being diagnosed (10). In South America, people living in areas where EEEV circulation occurs are usually more isolated, are of a lower socio-economic level, and have less access to health care (48, 53). Even when they have access to the health care system, there might not be great interest (since it does not impact treatment) or the capacity to diagnose the arbovirus causing encephalitis symptoms down to the specific viral agent (48). EEEV is not a reportable disease in South America (it is not highly communicable and does not seem to cause high numbers of clinical diseases) as it is in the US for example, making it more challenging to track numbers of reported EEEV diseases in South America.

Other suggested explanation is that the ecology of the virus in South America is such that would limit human-vector contact (6, 10). Low mobility of putative hosts (mainly small mammals) limits the spread of viruses and increases viral genetic diversity (4), and there might be an increased diversity of host species in South America (4). All of these ecological characteristics could account in part for less overt clinical disease due to EEEV in South America. However, there have been reports of more opportunities for increased human contact with viral habitats, and also reports of sero-prevalence studies in people with high close contact with forested areas like inhabitants of the Amazonian
region of Peru and Brazil without associated increase in the reported cases of human
disease of EEEV (52). In addition, in South America in areas like the Amazon, a lot of
modifications of the environment due to deforestation, agriculture practices, mineral
extraction, dam and road constructions (45, 54) are occurring which facilitates closer
contact with viral circulation habitats and increases the risk of human infection. Specific
vectors of EEEV are not well characterized in South America. *Culex* species are catholic
feeders but host preference and vectorial capacity studies for *Culex* *Melanoconion* in
South America are lacking (52). Therefore, there is the possibility that these surveys did
not look for EEEV in hosts or mosquito species that might be important for transmission
of the virus but have not been yet identified as such. In addition, small species of
mammals, the putative hosts for EEEV in addition to avian species, could present high
mortality due to EEEV infection and not be sampled during sero-surveys (42).

It has also been proposed that heterologous antibodies to other Alphaviruses
might confer some degree of cross-protection against EEEV (10). Lack of observed
clinical disease in South America cannot be attributed only to cross-protective effect of
infection with other Alphaviruses since close to 100% Alphavirus seroprevalence is not
observed and there is evidence of EEEV seropositivity in absence of any other
Alphavirus seropositivity (10). In Brazil, for example, in many of the serosurveys,
Flavivirus like Rocio, Saint Louis Encephalitis (SLE) and Yellow Fever virus were more
common than Alphaviruses (45, 47, 48, 53, 55). Another possibility is that the strain of
EEEV that circulates in South America is a milder one (56). Many biological
mechanisms of attenuation of the South American strain of EEEV have been proposed.
Antigenic differences (glycoproteins), have been observed between South and North
American EEEV, and humans in South America could actually be getting infected without developing any symptoms (10).

It has been suggested that EEE viral capsid protein might have a role in virulence due to its observed host cell gene expression inhibition (57, 58). This inhibition effect has been mapped to a twenty amino acid region (57). Some evidence that EEEV strains from North America are more resistant to the known general antiviral effect of interferon (INF) (10, 59) that seemed to be determined by both structural and non-structural viral protein gene regions (58). South American EEEV attenuation has also been proposed to be due to a vigorous response to and stimulation of IFN production. South American strain was capable of ameliorate disease due to North American EEEV strain either when administered prior to infection (immunization) or as a post-exposure agent. These studies have implications in new treatment avenues and in vaccine development (56-58).

North American and South American strains of EEEV differ by 25-38% at the nucleotide level (4). Conforming with the observed geographical, epidemiological, biological, ecological, and genetic differences between EEEV in North and South America, it has been recently proposed that the EEEV that circulates in South America is a distinct virus from the one that circulates in North America and should be re-classified based on the level of diversion between North American and South American strains of EEEV (60).

FINAL CONSIDERATIONS

EEEV circulates throughout the American continent with ecological and epidemiological differences between North and South America. The epidemiological and
ecological differences in EEEV between North and South America, and also within North America, enumerated in this paper highlight how focalized and unique EEEV transmission is and how these differences and similarities should be taken into account when considering and addressing control and preventive measures. EEEV infection in North America has serious health implications due to its associated severe morbidity and high mortality. In addition, in North America, people are moving into swamp lands, getting closer and closer to the virus habitat, increasing the risk of possible EEEV outbreaks. Although in South America a significantly lower number of cases, and associated human morbidity and mortality are reported, the concern still exists that with changing environments (population movement and destruction of natural habitats where viral circulation occurs), and due to EEEV being an RNA virus (prone to higher rate of mutation), overtime, EEEV might evolve to a more virulent form and become an arbovirus of public health importance in South America too. Since there is no FDA approved vaccine available for humans or specific treatment for EEEV infection, better understanding of the ecology of EEEV will help plan prevention and control measures. Therefore, there is a need for more studies into the ecology and epidemiology of EEE virus in North America (during both epidemic and inter-epidemic periods), and continued surveillance (in both South and North America) to better understand remaining questions in EEE virus ecology and epidemiology, and to help prevent possible outbreaks.
REFERENCES


35. Romano-Lieber NS, Iversson LB: [Serological survey on arbovirus infection in residents of an ecological reserve]. Rev Saude Publica 2000; 34(3):236-42


42. de Souza Lopes O, de Abreu Sacchetta L: Epidemiological studies on eastern equine encephalitis virus in Sao Paulo, Brazil. Rev Inst Med Trop Sao Paulo 1974; 16(5):253-8


48. Iversson LB, da Rosa AP, de Rosa JT: [Serological studies in research on arbovirus antibodies in the human population of the Ribeira Valley region. II -

49. Iversson LB, Travassos da Rosa AP, Travassos da Rosa JF, Pinto GH, Macedo O: [Serological studies in research on arbovirus antibodies in a human population of the Ribeira Valley region. IV - Survey of school children living in Iguape County, SP (Brazil)]. Rev Saude Publica 1983; 17(6):423-35


56. Gardner CL, Yin J, Burke CW, Klimstra WB, Ryman KD: Type I interferon induction is correlated with attenuation of a South American eastern equine encephalitis virus strain in mice. Virology 2009; 390(2):338-47


SUMMARY CONCLUSIONS

Discussion and Conclusions

In our host preference study, the most commonly targeted species in blood meal counts were white-tailed deer (608), Great Blue Heron (135), cotton mouth (86), Yellow-crowned Night Heron (72), human (41), bull frog (33), Northern Cardinal (32), Green Heron (22) and American bittern (22). White-tailed deer, Great Blue Heron, Yellow-crowned Night Heron, Northern Cardinal, and Green Heron blood meals were seen in every year of the study. Blood meals for the top hosts in blood meal counts for all years combined were identified from multiple mosquito species. Host blood meals from the most commonly targeted avian species (Great Blue Heron, Green Heron, Northern Cardinal, and Yellow-crowned Night Heron) came from Culiseta, Aedes, and Culex mosquitoes. White-tailed Deer blood meals came from nine different species of mosquitoes including those in the genera Aedes, Anopheles, Coquillettidia, and Culex. Culex erraticus originated blood meals made the vast majority of identified blood meals for all five top host species, ranging from 65.6% to 94.4%.

We looked at the overall feeding pattern of viral positive mosquitoes species by submitting them to our statistical analysis of randomness and interaction, blood meals from mosquito species from which we had a virus positive isolate throughout the study period. Those species were Cx. erraticus, Cx. peccator, Cx. territans, Cs. melanura, Ae.
Avian blood meals showed a p-value of 0.008 on Runs test, and a p-value of 0.115 on Tukey’s one degree of freedom test indicating avian blood meals counts were not random and were consistent over the observed time. Mammal blood meal counts had a p-value of <0.001 in Runs test, but a p-value of 0.002 on the Tukey’s test. However, when we look at the graph for mammalian blood meal counts over time for the studied years, we can see that the lines cross (what is the graphical representation of interaction) but seem to follow the same pattern with a lag, a pattern that is more prominent for 2006 and 2007.

Amphibian and reptile species have been represented in blood meal analysis and serological studies in areas of arboviral transmission [14, 15, 45-47]. Given the possibility that amphibians and reptiles might play a role in EEEV transmission either as possible local hosts in which EEEV could be over-wintering [14, 15, 46], or as a source of infection to predator birds that feed on them [45], we looked at amphibian (total of 171 blood meals is four years) and reptile (total of 175 blood meals identified in the four years of study) blood meals from 2004, 2006-2008, using the statistical test Runs and Tukey’s. Most frequent reptile species represented in blood meals were cottonmouth (87), green anole (28), and plain-bellied water snake (17), however, green anole blood meals were detected only in 3/4 years of study. The most common species of amphibians found in blood meals were bullfrog (40), green frog (28), and Spring peeper (21), but only bullfrog blood meals were observed in all four years.

Runs test for amphibians blood meal was 0.003, for reptiles was 0.04, and for amphibians/reptiles combined was 0.002, leading us to reject the null hypothesis of random order even though the p-value for reptiles blood meal on runs test (0.04) barely
reached statistical significance at $\alpha = 0.05$. P-values from Tukey’s test were 0.056 for amphibians, 0.0000007 for reptiles, and 0.02 for amphibians/reptiles combined. These p-values suggest that blood meals from amphibians at the TNF site, Alabama, for 2004, 2006-2008 were consistent from year to year. Again, a p-value of 0.056 is very close to the set level for statistical significance ($\alpha = 0.05$). As data on amphibian and reptile blood meals for more years become available, it would be interesting to add them to this analysis and observe if the pattern suggested by this preliminary statistical testing can be verified.

For Yellow-crowned Night Heron and Northern Cardinal, Runs test values lead us to conclude that blood meal counts for these avian hosts are a random occurrence. Hassan, et. al 2003 report Yellow-crowned Night Heron as an over-represented and Northern Cardinal as an under-represented avian host species at the TNF site for 2002 [30]. In our study, even though total blood meal counts for Yellow-crowned Night Heron and Northern Cardinal were smaller and more sparsely distributed than blood meal counts for Great Blue Heron and White-tailed Deer, we do not believe that the smaller sample size decisively impacted our power to detect a significant p-value and reject the Runs test null hypothesis of randomness since Runs test is a non-parametric test (does not assume a specific distribution) thus being more suitable for small size samples. In addition, when we re-run the Runs test, including only the 2003 Yellow-crowned Night Heron blood meal counts, we observed a p-value of 0.083, which is smaller but still leading us to call the Yellow-crowned Night Heron blood meal counts a random event for that year. Combined data for all six years for Yellow-crowned Night Heron and Northern Cardinal when tested by Tukey’s 1-way also indicate that blood meal counts from these avian
species do not show a year-to-year consistent pattern (p-value <0.001 for both species). However, Yellow-crowned Night Heron blood meals have been identified from three different mosquito species, two of which are known to be competent vectors of EEEV, *Cs melanura* and *Cx erraticus* [21], and another species, *Cx peccator*, that is a more generalist feeder (has the potential to serve as a bridge vector in epizootic transmission) [15]. Thus more research is needed to investigate the role of Yellow-crowned Night Heron in the ecology of EEEV in Alabama given that statistical significance does not always translate into biological significance.

Runs test results suggest that blood meal counts for Great Blue Heron and White-tailed Deer were not generated from a random process. In addition, they seem to reflect an avian to mammal class shift in host preference. This host shift has been reported for another arbovirus, West Nile virus, where robins are the important avian host early in the season, and humans become the main host later in the transmission season [13, 27].

Great Blue Heron has been previously reported as a preferable feed upon species by possible mosquito vectors of EEEV. However, this possible preference by EEEV vectors has been largely attributed to this avian species size and less to vector preference per se, and to its low irritability to mosquito bites [21, 30, 37].

Many studies have reported seroprevalence of EEEV and other arboviruses in deer [38-40], White-tailed Deer as a source of blood meals from *Culiseta* [41], deer with confirmed EEEV infection, high seroprevalence for EEEV, and clinical EEEV disease [42, 43]. A fatal case of extensively laboratory confirmed (using different methods such as histopathology, immunohistochemistry, cell culture, and RT-PCR) EEEV infection in a white-tailed deer in Houston County, Georgia, in 2001, triggered a seroprevalence
study of EEEV in hunter-killed white-tailed deer sera from that area. Fourteen percent of
the deer samples were found seropositive for EEEV antibodies (ages 1.5 to 4.5 years),
and some more localized areas had 32% and 55% deer seroprevalence for EEEV. In
addition, this fatal deer case preceded two horse cases in the same region, a known
enzootic area for EEEV, and one human case of EEEV was reported in an adjacent
county in that same year [42]. A passive surveillance (cases were reported by the local
population) for Chronic Wasting Disease (CWD) in Michigan identified seven confirmed
(based on PCR and pathology investigations) EEEV infection out of thirty deer with
clinical signs of CWD. In that same study, molecular epidemiological linkage (through
virus isolation and sequence) was determined between two deer samples in the passive
surveillance and reported EEEV horse cases in the same area. In addition, in the area
surrounding the deer cases, preceded by few weeks, four equine EEEV infections were
diagnosed [43].

In light of these recent reports and of our own findings, the role of deer in the
ecology of EEEV in the areas where they are abundant and most importantly where they
have been found seropositive for EEEV, or in high frequency in the identified blood
meal, or with clinical signs of EEEV disease is still to be determined. For example, one
important question is if these findings related to deer are a consequence of its abundance
in those areas or of some arbovirus vector activity. Many factors can contribute to deer
being a more targeted specie; for example their big size, their high body mass, their
behavior towards mosquito biting (irritability), or a preference for this host by mosquito
species involved in EEEV transmission. If, in further studies, host preference towards
deer could be determined in those areas, that will have an impact on the eco-
epidemiology of EEEV with implications in prevention/control measures.

These results might suggest that the profile seen in the feeding patterns upon
Great Blue Heron and White-tailed Deer may reflect an underlying biological process.
On the other hand, it may be possible that they just represent opportunistic feeding in a
large size, abundant animal in the area rather than host preference per se. In any case,
reports of increased seroprevalence and signs of clinical disease to EEEV in deer warrant
more studies into the role these two species play in the ecology of EEEV in Alabama and
elsewhere based on their observed temporal pattern. Further studies exploring the life
events of deers and Great-Blue herons in those areas in relation to the behavior of
mosquito species in the area will help us better understand a possible biological basis for
the observed temporal pattern.

Many reasons have been cited as possible explanations for the differences in
EEEV reported disease rates between North and South America. The fact that the
number of reported human EEEV infections in South America compared to North
America is much lower has been attributed in part to less surveillance efforts or less
sensitive surveillance for this virus in South America [11]. However, there has been a lot
of surveys for EEEV in South America, looking for EEEV antibodies in the sera and
virus in blood or tissues of possible hosts, and for EEEV in possible vectors in South
American countries such as Brazil, Argentina, Trinidad, Venezuela and Peru. These
surveys, even when found EEEV positive antibodies or isolated the virus from those
samples, failed to find reports of humans presenting with clinical disease for EEEV. In
cases when EEEV was not found in those areas, other arboviruses that cause disease in humans were detected.

Symptoms of EEEV are very unspecific and can be presenting in the same way as other arboviruses in the area. In South America, a great number of arboviruses co-exist, so it is possible that some of the human cases identified clinically or with a less specific laboratory assay as of other arboviruses could actually be due to EEEV infection [48-54]. It has also been mentioned that heterologous antibodies to other Alphaviruses might confer some degree of cross-protection against EEEV [6]. In Brazil, for example, in many of the serosurveys, Flavivirus like Rocio and Saint Louis Encephalitis (SLE) were more common than Alphaviruses [50-53, 55].

EEEV occurs in a localized area, and epizootics occur at certain time intervals. Human cases could be occurring but not being diagnosed [6]. In South America, people living in areas where EEEV circulation occurs are usually more isolated, are of a lower socio-economic level, and have less access to health care [50]. Even when they have access to the health care system, usually there is not the interest (since it does not impact treatment) or the capacity to diagnose the arbovirus causing encephalitis symptoms down to the specific viral agent [50].

Another possibility is that the strain of EEEV that circulates in South America is a milder one [56]. South American strains are less virulent due to antigenic differences (glicoproteins), and humans in South America could actually be infected without developing any symptoms [6]. It has recently been demonstrated that the two different strains of EEEV trigger different immune responses mediated by type I interferon [56].
Other suggested explanation is that the ecology of the virus in South America is such that would limit human vector contact [6, 7]. However, there have been reports of more opportunities for increased human contact with viral habitats, and also reports of seroprevalence studies in people with high close contact with swamps and forested areas like inhabitants of the Amazonian region of Peru and Brazil without associated increase in the reported cases of human disease of EEEV [11]. However, in South America in areas like the Amazon, a lot of modifications of the environment due to deforestation, agriculture practices, mineral extraction, dam and road constructions [55, 57] are occurring which puts people in closer contact with viral circulation habitats and increases the risk of human infection.

In addition, specific vectors of EEEV are not well characterized in South America since we have little knowledge about the ecology and epidemiology of EEEV. Therefore there is the possibility that these surveys did not look for EEEV in animals or mosquito species that are important for transmission of the virus but have not been yet identified as such.

Disturbances of the natural habitat of the virus could have opposite effects such as either make the virus disappear by diminishing the number of susceptible hosts to keep the transmission going, or work as a pressure factor that can make the virus adapt to cause enough high viremia in humans and enable human-to-human transmission which happened with YF and Dengue (also RNA arborviruses like EEEV with a higher rate of mutation due to the lack of replication proof reading enzymes) [1, 8, 57].
Strengths and Limitations

Blood meal analysis can be biased depending on the mosquito collection method since one specific method can enrich samples with one specific mosquito species (based on its behavior) or mosquitoes of one physiological stage (for example, blood engorged females) [8]. This study applied a combination of a variety of methods of mosquito collection including resting boxes that are more suitable for female engorged mosquito (sources of blood meal for identification).

The ecology of EEEV is very complex and involves many variables relating to the vector, the virus, hosts, and the environment. All these factors interact to make the bigger picture and not all of those variables have been identified and/or measured in this study (or any other study for that matter). However, the data analyzed here and collected over time by Dr. Unnasch's group constitutes of one of the largest and most complete datasets of this kind.

Generalization of the results obtained from the analysis of the present data might not be possible since it represents collection of data from a single site, TNF in Alabama. In addition, the ecology of EEEV has been shown to be very localized and unique to certain areas. However, the analytical approach developed here can be applied to other locations and other arboviruses. To our knowledge, this is the first study to use statistical approach to test observed class shifts in host preference of arboviruses like EEEV, the agent of an important arboviral disease in the Americas.

The majority of the serological surveys performed in Latin American countries reported here to find the sero-prevalence of EEEV in human, horses, mosquitoes and other animals was done in a time where the available techniques were less sensitive than
the techniques available today. Even though their results have been consistently repeated by others even in more recent years, their sensitivity and specificity should be taken into account when analyzing and generalizing their findings. Due to the low level of antibody response to EEEV in South America and to the unknown duration of circulating antibodies to EEEV following infection, a molecular assay such as PCR might be more suitable to find infected individuals in this area than serological assays [6].

Public Health Implications

EEEV infection has very serious health implications due to its high morbidity and mortality. EEEV outbreaks are increasing due to population movement: people are moving into swamplands, and getting closer and closer to the virus habitat. In addition, destruction of viral habitats for housing, dams and road construction, and mineral extraction (for instance, in the Amazon region) all contribute for increased risk of human arboviral infection [55, 57]. Since there is an FDA approved vaccine available only for horses, but not for humans at this time, better understanding of the ecology of EEEV will help prevention and control measures. More surveillance and work is needed in both North America and South America including during the inter-epidemic periods in order to better understand the ecology and epidemiology of EEEV. The methodology presented in this work can be used for study issues of temporality and host preference in EEEV in other sites, as well as other arboviruses.


