Eco-Epidemiology of Eastern Equine Encephalitis Virus

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Eco-Epidemiology of Eastern Equine Encephalitis Virus

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

To my loving wife Kelsey, without her support this would not have been possible.
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ABSTRACT

Eastern Equine Encephalitis virus (EEEV) is an alphavirus with high pathogenicity in both humans and horses. Florida continues to have the highest occurrence of human cases in the USA, with four fatalities recorded in 2010. Unlike other states, Florida supports year-round EEEV transmission. This research uses Geographic Information Science (GIS) to examine spatial patterns of documented sentinel seroconversions and horse cases in order to understand the relationships between habitat and transmission intensity of EEEV in Florida. Sentinel sites were categorized as enzootic, periodically enzootic, and negative based on the amount of chicken seroconversions to EEEV. Sentinel sites were analyzed based on land classification data using the Kruskal-Wallis test to determine which habitats were associated with disease transmission. Cluster analyses were performed for the horse cases using density-based spatial clustering of applications with noise (DBSCAN). Ecological associations of EEEV were examined using compositional analysis and Euclidean distance analysis to determine if the proportion or proximity of certain habitats played a role in transmission. The research in these studies provides evidence of ecological associations for EEEV transmission in Florida that hasn’t been previously analyzed. Furthermore, these studies provide the groundwork for better understanding of why there is a disproportionate number of horse and human cases of EEEV in Florida than in any other state.
INTRODUCTION

An arthropod-borne virus (arbovirus) is a virus that requires a hematophagous (blood-sucking) arthropod vector for transmission into vertebrate hosts to maintain its life cycle [1]. There are 23 different types of zoonotic viral diseases that make up the arbovirus group; however, those that are medically important belong to three different families, Togaviridae, Flaviviridae, and Bunyaviridae. Out of the 23 different arboviruses half are found within the United States.

Historically, the five major arboviruses that are important in the United States are Eastern Equine Encephalomyelitis virus (EEEV), St. Louis Encephalomyelitis virus (SLEV), La Crosse Encephalomyelitis virus (LAC), Western Equine Encephalomyelitis virus (WEEV) and West Nile Encephalomyelitis virus (WNV) [2]. The encephalitis viruses are considered important because their infection often causes neurological damage or death [3]. In the past few years, dengue virus (DENV) has reemerged in Florida; consequently, dengue is now considered an arbovirus of importance to the United States.

The arbovirus transmission cycle is considered complex due to the necessary interactions between the vertebrate host and the disease vector. The basic transmission cycle in arboviruses is through an adult mosquito (vector) feeding on an infected host. The mosquitoes then carry the infective viral particles in their salivary glands and upon their secondary blood feeding transmit the virus to other susceptible hosts [4]. Many
factors contribute to the host/vector interaction and each to a varying degree increases or decreases the chance of virus transmission.

Reservoir competence is the capability of the host to amplify enough virus in their system to be infectious without it being fatal to them. Reservoir competence is important to the transmission cycle in that it provides the virus a host in which it can efficiently replicate. Many of the arboviruses share common reservoir hosts. Several species of birds are considered competent reservoir hosts for EEEV, WEEV, SLE, and WNV [5, 6]. For the non-avian cycle arboviruses (LAC) the vertebrae amplifying host is chipmunks and tree squirrels [7, 8]. The dengue virus is unique in this group in that its host is humans (endemic genotypes) and primates (sylvatic genotypes) [3]. There are also several hosts that are considered dead end hosts since they do not have the reservoir competence to amplify the virus to infectious levels before the disease becomes fatal. In EEE, dead end hosts include humans, horses and some birds (pheasants, quail, and ostriches). For WNV, these dead end hosts are mammals.

For viral transmission to be successful, a competent vector is also needed. Vector competence is the ability of an insect to receive, carry and transmit virus to another host. There are many factors that affect vector competence, temperature [9], population dynamics [10, 11], and the concentration of viral particles in the blood meal [12, 13]. The interaction between competent vectors and competent hosts is an important element in determining vectorial capacity. Vectorial capacity is determined by the density of vectors, the competency of the vector species, the ability to successfully feed off of a viremic host, the incubation time needed to render the infected vector infectious to other hosts, and the daily survival rate [14]. Competent arbovirus vectors are often different
based on geographical locations and virus strains. The geographical spread of arboviruses within the United States is not uniform and has differing ecological habitats. It is the interaction among the vectors and reservoir hosts within their respective environments that largely contributes to the existence and maintenance of arboviruses.

Eastern Equine Encephalitis virus (EEEV; family Togaviridae, genus Alphavirus) is a pathogenic arbovirus with a high case fatality rate among infected humans and horses. Human symptomatic cases of EEE exhibit a case fatality rate of 35% or greater, and many survivors suffer residual neurological sequelae which can result in million dollar health care costs per individual [7, 15, 16]. EEEV is endemic in many of the states of the Eastern USA with cases of neuro-invasive EEE reported in 20 states [17]. The demographic with the highest risk for fatal EEEV infections are the very young and the old. Symptoms of EEEV include headache, fever, chills, vomiting, mental disorientation, seizures, and coma [18]. There is no approved human vaccine and no treatment available for those infected with EEEV. Prevention and surveillance still remain the only options for controlling the risk of infection to humans.

In North America, EEEV circulates in a mosquito-avian cycle, usually within freshwater swamps [19]. It is believed that the interaction between the highly efficient enzootic vector Culiseta melanura and competent avian reservoirs in these swamps creates an ideal situation for amplification and maintenance of the virus. Primary reservoir hosts for EEEV in North America are migratory passerine songbirds, starlings, and wading birds. Once infected, these passerine birds spread the virus to surrounding areas through migration and foraging behaviors [7].
EEE is a reportable human and veterinary disease in the United States [20]. Florida’s first documented human case of EEE was in 1952 [17]. Since 1964, human cases of EEEV in Florida have accounted for 25% of the total fatalities observed due to EEEV in the United States [21]. Today, EEEV is widespread across Florida, with transmission reported in 64 of Florida’s 67 counties [22, 23]. Despite the availability of an equine vaccine, EEEV in Florida still claims an average of 70 horse fatalities a year.

In the Northeast and in the South Central states, epizootic outbreaks involving humans and horses peak in August and September [24]. In contrast, EEEV transmission in Florida occurs throughout the year, with most human and horse cases occurring in June and July [25]. Outbreaks can occur when the virus spreads into mosquito species that feed on a variety of hosts, not just birds. Bridge vectors are mosquitoes that feed on both birds and mammals and have the potential to transmit EEEV to humans and horses during epizootics. The bridge vectors of EEEV can vary between regions and habitats. In coastal areas, salt marsh mosquitoes such as Aedes sollicitans and Culex salinarius are thought to be important bridge vectors [26, 27]. At inland sites, a number of mosquito species have been implicated as bridge vectors, including Coquillettidia perturbans [28], Aedes vexans [27], and Culex erraticus [29, 30].

Studies conducted in the Southeastern United States have shown that high abundances of Cx. erraticus have been collected during peak seasons of EEEV resulting in high numbers of infected pools [31]. Culex erraticus often takes blood meals from a variety of hosts and has also been shown to feed on mammals, birds, reptiles, and amphibians [32]. Previous studies show that Cx. erraticus exhibits a feeding shift during the year. In early spring, Cx. erraticus prefers to feed on avian hosts while during the
summer it shifts to feeding on mammals [32]. The feeding shift to mammals in the summer coincides with the temporal pattern of horse cases of EEE.

*Culiseta melanura*, the principal enzootic vector of EEEV, can also be considered a bridge vector because even though it primarily feeds on avian hosts studies have shown that it will occasionally feed on mammals and reptiles [33-37]. Previous studies have shown that in Northeastern United States, *Culiseta melanura* is collected year round; however, vector abundance has been shown to peek in both early June and mid-August [38]. This contrasts recent studies done in Alabama’s Tuskegee National Forest in which *Cs. melanura* was rarely captured.

A study on the ecology of EEEV in the Southeastern United States found early season EEEV infection in reptile and amphibian feeding mosquitoes, which included *Culex territans*, *Uranotaenia sapphirina* and *Culex peccator* [39]. *Culex peccator*, from which EEEV has been repeatedly detected [40], takes around 54% of its bloodmeals from snakes [41]. *Culex peccator* and other suspected EEEV vectors spend the winter in the same habitat as snakes [42]. To test the hypothesis that snakes are competent hosts for EEEV, laboratory studies were conducted using garter snakes. Viremia within the garter snakes reached sufficient levels to infect *Cs. Melanura* [43]. The viremia in snakes was shown to exist after hibernation suggesting that snakes are capable of maintaining a circulating viremia through hibernation [43]. In examining the role snakes have in over-wintering EEEV, studies have shown that wild-caught snakes exhibit high seropositivity rates [44]. It is therefore plausible that vectors, in search of a convenient host, would encounter snakes in their over-wintering sites, by virtue of their proximity. The potential of this proximal encounter is more likely to occur in the early spring, when both
mosquitoes and snakes become active [42]. While it has not been proven that snakes serve as natural over-wintering reservoirs for EEEV, there is evidence that supports the possibility of this hypothesis [45].

Arbovirus surveillance in Florida is made up of many different agencies including Florida Department of Health, Department of Agriculture and Consumer Services, and many county based mosquito control districts. The Florida Sentinel Chicken Arbovirus Surveillance Program was initially created in 1978 due to frequent outbreaks of St. Louis encephalitis virus (SLE) [46, 47]. Sentinel chickens have been shown to be good early detector of EEEV and other arboviruses before cases in humans and horses occur [48]. Currently, arbovirus surveillance in Florida is conducted in 33 of the 67 counties. Many of the Florida sentinel chicken sites are still placed according to SLE and West Nile virus risk areas and few are placed according to EEEV risk areas. The lack of EEEV based sentinel site placement is partially attributed to the limited understanding of at risk habitats. Deciphering the ecological associations of EEEV is essential to more efficiently and accurately monitor EEEV for public health.

Landscape and spatial epidemiology are important variables to consider when investigating the connection between pathogens and their hosts, vectors, and the environment [49, 50]. Synergistic elements are often involved in obtaining and maintaining an optimal disease transmission cycle; some of these elements include appropriate habitats for vector and host reproduction, population densities, host vector proximity, and meteorological influences.
The advancement of remote sensing technology and geographic information science (GIS) has given researchers a greater opportunity to explore these habitat elements and their role in vector-borne disease transmission [51-55]. The use of global positioning systems (GPS) in combination with GIS allows researchers to collect spatially linked data and provides a means of monitoring pathogens across time and space through varying environmental landscapes.

In studying arboviruses, the habitat structure is based on many factors influencing the local ecosystem. The vegetation density that provides breeding habitats for both vector and host populations can be determined through aerial photography or satellite imagery. Spectral distinction of water and vegetation densities allows remote sensing to identify potential larval habitats [56]. This is critical in determining optimal areas for monitoring because many mosquito species have specific questing behaviors to locate blood meals in order to lay eggs [57]. The ability to reduce the area for vector monitoring will increase the feasibility of studying arboviruses over large ecological regions.

Many mosquito species have habitat preferences and are often focused around specific landscape areas. Analyzing habitat preferences would be an important data variable to consider based on the arbovirus of interest. In studying West Nile virus, the habitat of focus would be urban areas since transmission is negatively associated with rural and agricultural areas [58]. In Connecticut, logistic regression models were used to determine the habitat suitability of West Nile vectors and the corresponding vector abundance. The results showed that Cx. pipiens was most abundant with urban areas, Ae. vexans was more abundant around grasslands and agriculture, and Cs. melanura
abundance was positively associated with deciduous forests [59]. Studies conducted in the Northeastern United States have also shown that the vegetation density within an urban area increases the risk of West Nile transmission [60]. Implementing GIS as a tool to analyze habitat preferences for EEEV has been used in the Northeastern United States. Analyzing habitat composition in relation to mosquito species abundance resulted in the identification of wetlands as a major contributor to variances in Cs. melanura populations [19]. The traditional habitat of importance for EEEV is hardwood swamps since that is the breeding habitat of the enzootic vector Cs. melanura [19]. However, studies have shown that EEEV infected Cs. melanura have been detected in residential woodlands and away from the typical hardwood swamps [61] suggesting that local populations of Cs. melanura can be implicated in transmitting EEEV.

Our study focuses on understanding the ecological variables associated with EEEV transmission in Florida. We wanted to determine which ecological variables play a role in the avian transmission cycle as well as the mammalian transmission cycle. We hypothesized that ecological associations could be determined through analyzing the Florida sentinel chicken surveillance program which is used by the state to monitor arbovirus transmission. Our hypothesis was that through comparing EEE negative sentinel sites with EEE positive sites habitat associations would arise. We also wanted to look at the mosquito distribution at these sights based on habitat classifications in order to determine if EEEV vectors are more abundant in specific habitats.

The second aim of this research was to determine the spatial epidemiology of EEE in horses throughout Florida using documented horse cases. Horse case locations were retrieved from the Florida Department of Health and habitat associations were
assessed using GIS. We hypothesized that the ecological associations found from the horse cases would show a common habitat coinciding with the sentinel site analyses. Disease incidence coupled with regional and local clustering was analyzed to determine if there were regional differences in EEE transmission within the state of Florida. We hypothesized that the regional distribution of disease would be correlated to the regional habitat distinctions in Florida.

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CHAPTER 1

Habitat associations of Eastern Equine Encephalitis transmission in Walton County
Florida

Note to Reader

This chapter has been previously published (Vander Kelen, P.T., et al., Habitat
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Abstract

Eastern Equine Encephalitis virus (EEEV; family Togaviridae, genus Alphavirus) a highly pathogenic mosquito-borne virus is endemic to eastern North America. The ecology of EEEV in Florida differs from that in other parts of the USA. EEEV in the Northeastern USA is historically associated with freshwater wetlands; however no formal test of habitat associations of EEE in Florida has been reported. Geographical Information Sciences (GIS) was used in conjunction with sentinel chicken EEEV seroconversion rate data as a means to examine landscape features associated with EEEV transmission in Walton County, Florida. Sentinel sites were categorized as enzootic, periodically enzootic, and negative based on the amount of chicken seroconversions to EEEV from 2005-2009. EEEV transmission was then categorized by land cover usage using Arc GIS 9.3. The land classification data was analyzed using the Kruskal-Wallis
test for each land us class to determine which habitats may be associated with virus transmission (sentinel seroconversions). The habitat class found to be most significantly associated with EEEV transmission was tree plantations. The ecological factor associated with reduced levels of EEEV transmission were vegetated non-forest wetlands. *Culiseta melanura*, the species generally considered to be the major enzootic vector for EEEV was relatively evenly distributed across all habitat classes, while *Aedes vexans* and *Anopheles crucians* were most commonly associated with tree plantation habitat.

**Introduction**

Eastern Equine Encephalitis virus (EEEV; family *Togaviridae*, genus *Alphavirus*) is a pathogenic arbovirus with a high case fatality rate among infected humans and horses. Human symptomatic cases of EEE exhibit a case fatality rate of roughly 50%, and many survivors suffer residual neurological sequelae [7, 15]. EEEV is endemic in many of the states of the Eastern USA with cases of neuro-invasive EEE reported in 20 states [17]. Since 1964, human cases of EEEV in Florida have accounted for 25% of the total fatalities observed due to EEEV in the United States [21].

EEEV is widespread across Florida, with transmission reported in 64 of Florida’s 67 counties [22, 23]. Studies of EEEV in Florida suggest that its ecology is both more diverse and distinct from that seen in other parts of the USA. For example, EEEV circulates year-round in Florida, while it is dormant during the winter months elsewhere [62, 63]. EEEV isolates from Florida appear to be genetically similar to those found in the northeastern USA [64-68]. However, specific ecological niches may exist that enhance amplification and transmission of EEEV in Florida. For example, higher
sentinel chicken seroconversion rates have been reported over the last several years in Florida’s panhandle region than in the southern part of the state [69].

Most counties in Florida maintain active surveillance and vector control programs aimed at reducing the threat of arboviruses. These programs rely upon mosquito collections to monitor levels of probable vectors and serological surveillance of sentinel chickens to detect arboviral transmission. Historically, sentinel chicken flock locations were determined based upon the proximity to human cases of St. Louis encephalitis virus (SLEV) during the last major human outbreak of SLEV in Florida, which occurred during the 1970’s [46, 47]. Subsequently, these flocks were used for surveillance of West Nile virus (WNV), a flavivirus related to SLEV, that was introduced to the United States in 1999, and to Florida in 2001 [48]. While the ecological predictors associated with WNV transmission have been extensively studied [49, 70-73], ecological factors influencing the spread of EEEV have not been fully defined, particularly in Florida. As a result, Florida’s sentinel chicken surveillance and vector control programs have limited information for placement of surveillance sites for monitoring EEEV and a more targeted approach would improve the efficacy for detection of EEEV activity.

Landscape and spatial epidemiology are important variables to consider when investigating the connection between pathogens and their hosts, vectors, and the environment [49, 50]. Synergistic elements are involved in obtaining an optimal disease transmission cycle; some of these elements include appropriate habitats for vector and host reproduction, population densities, and meteorological influences. The advancement of remote sensing technology and geographic information science (GIS) has given researchers a greater opportunity to explore these habitat elements and their role in
vector-borne disease transmission [51-55]. With respect to EEEV, remote sensing and GIS have been successfully utilized to characterize associations between vector abundance and landscape variables in the Northeastern USA [19].

For the purpose of this study, GIS was used in conjunction with sentinel chicken EEEV seroconversion rate data as a means to examine the landscape features associated with EEEV transmission within the Florida panhandle region (Walton County). Our investigation thus focused on determining landscape risk factors associated with elevated EEEV transmission as monitored by seroconversions among sentinel chicken flocks.

**Materials and Methods**

The sites selected for inclusion in this study were in Walton County, located in the Western Panhandle region of Florida (Figure 1). The county’s landscape is dominated by five major land cover classes, which together account for 86% of the total county area; these habitats include upland forests, tree plantations, wetlands, cropland, and residential (Table 1). Walton County was chosen for several reasons. First, an examination of records maintained by the state of Florida over the past five years have suggested that Walton County is one of the most active in the state in terms of EEEV activity [69]. Second, Walton County has consistently participated in the statewide arbovirus surveillance program that monitors sentinel chickens for disease activity.

Twenty four (24) sentinel sites were selected for inclusion in the study from the possible 29 sites in the county. Five sites were excluded from this study because they were only operational for one year, and therefore comparisons of yearly virus activity during 2005-2009 were not possible at these sites. Sentinel flocks in areas surrounding
Choctawhatchee Bay were maintained by South Walton Mosquito Control and utilized 2-3 chickens per flock. Sentinel flocks of inland areas of the county were maintained by North Walton Mosquito Control and utilized 6 birds per flock. Chickens in both districts were maintained in the field in cages that protect them from predators and weather. Blood samples from the sentinels were collected weekly by county mosquito district personnel and then shipped to the Florida Department of Health Bureau of Laboratories for testing. Sera were screened using an hemagglutination inhibition (HI) assay to detect EEEV antibodies [74]. If the sentinel sera HI assay was positive, a confirmatory test for IgM antibody to EEEV was then conducted as previously described [7, 15]. Chickens that were confirmed EEEV positive were removed from the flock and replaced with a serologically naïve bird.

Sentinel sites were categorized based on the number of chicken seroconversions to EEEV from 2005-2009. Sites with at least one confirmed positive EEEV seroconversion per year for three or more of the last five years were classified as EEEV enzootic sites. Sites with at least one EEEV positive sentinel per year for less than three of the last five years were classified as periodically EEEV enzootic. Finally, those sites with no EEEV seroconversions were classified as EEEV negative (Figure 2). Seroconversion rates were calculated by dividing the total amount of confirmed seroconversions per site by the number of susceptible chickens exposed at that site during the time period.

The habitats at the chosen sites were characterized using the level two land cover usage classifications taken from the Northwest Florida Water Management District Land Use Land Cover 2004 [75]. In addition, a habitat analysis was conducted using Arc GIS
9.3 on all 24 sites. The Florida Department of Environmental Protection's Bureau of Watershed Restoration has developed land use and land cover maps using Digital Ortho Quarter Quad Aerial Imagery program Color Infrared and True Color photography [75].

Habitat descriptions for the land use land cover are based on the Florida Department of Transportation schema and encompass four different levels, with level 1 being the most basic and level 4 the most specific [76]. Level 2 descriptions were used in this study because they differentiated between wetland types (Figure 3). Out of the 42 sub-classifications found in the level 2 categories, 11 were used in this study, which were selected based on their habitat importance to mosquito vectors associated with EEEV or possible hosts. Several of the classes were combined, such as lakes, streams, and reservoirs. The remaining classes were excluded because they were not suitable habitats for EEEV (e.g. large paved areas, beaches, ocean, and airports).

Previous studies have shown that the average mosquito dispersal distance is between 1-3km [31, 77-79]. Therefore, a 1.5 kilometer buffer was created around the chicken sentinel sites to account for average mosquito movement during the collection period. Habitat features were then extracted from each buffer area using the intersect function and acreage composition was summarized. The land classification data was analyzed using the Kruskal-Wallis test [80] for each land use class to determine which habitats may be associated with virus transmission and comparisons were made between enzootic, periodically enzootic, and negative sites.

Scatter plots were produced with best-fit lines, to visualize the relationships between selected habitat covariates and EEEV seroconversions rates of chickens in
sentinel flocks. Six of the eleven total habitat types were selected \textit{a-priori}, which were predicted to have the greatest potential for biological influence on transmission potential of EEEV.

Mosquitoes were collected twice weekly from May through August 2009 at all 24 sites using CO$_2$-baited light traps. Upon collection, mosquitoes were identified using morphological keys [81]. Mosquitoes were pooled by species, collection date and site, with each pool containing a maximum of 50 individuals. Pools were tested for the presence of EEEV using a real time RT-PCR assay [82, 83]. Positive mosquito pool homogenates were centrifuged and supernatant (ca. 1ml) was collected, filtered and added to confluent Vero cells in individual T-25 flasks for virus isolation, as previously described [84]. Mosquito collections were normalized by trap night across habitats to determine relative species abundance within habitat types during the collection period.

**Results**

Of the 24 sites in Walton County included in the study, 9 were classified enzootic, 10 were periodically enzootic, and 5 were negative using the criteria described in Materials and Methods. The difference in spatial extent of each land cover type among the three EEEV classes was statistically compared. These data were first examined for homogeneity of variance and normality, two critical assumptions of normal parametric statistics. These tests revealed that the data strongly violated each of these assumptions, with variance ratios exceeding 5 and highly significant deviations from normality in the Kolmogorov-Smirnov tests in 92% of the cases. As a result of these violations, differences were tested using the Kruskal-Wallis test, a non-parametric equivalent of a one-way ANOVA. The Kruskal-Wallis test results suggested significant associations in
particular habitats for EEEV activity (Table 2). Three habitat classifications (cropland and pastureland, tree plantations and vegetated non-forested wetland) were significantly associated with EEEV activity ($p < 0.05$) while three (lakes and streams, upland coniferous forest and wetland forested mixed) were marginally significantly associated with EEEV activity ($0.05 < p < 0.1$).

Scatter plots were then prepared comparing the incidence of seroconversions to the number of acres of each habitat for the habitats shown to be significantly or marginally significantly associated with EEEV activity (Figure 4). Wetland mixed forest and tree plantation habitats were found to be positively associated with EEEV transmission. For example, enzootic sites had an average of 303.36 acres of tree plantation habitat per site, while the negative sites averaged only 22.69 acres per site (Table 2). Two habitat classes (cropland and pastureland, and lakes and rivers) had little or no association with EEEV activity when analyzed by scatter plot. This divergence in the results produced in the scatter plot and non-parametric analyses was at least in part due to the bimodal distribution in habitats among the three EEEV activity classification groups. For example, cropland and pastureland were present in similar amounts in enzootic and negative sites (102.3 and 161.5 acres respectively) while periodically enzootic sites averaged only 4.98 acres of this habitat (Table 2). The two remaining classes (upland coniferous forest, and vegetated non-forested wetland) were negatively associated EEEV transmission to varying degrees (Figure 4).

A total of 242 chickens seroconverted in Walton County from 2005-2009 (Table 7). The average EEEV seroconversion rate in the sentinel chickens across all sites over the study period was 18.5%, with the most active sentinel flock averaging a
seroconversion rate of 42.2%. During 2009, 64 sentinel chickens seroconverted in Walton Co, resulting in an 18.7% overall seroconversion rate (Table 3). Two enzootic sites in 2009 had high seroconversion rates (58.6% and 45.8%) which were greater than the five year average rate of 42.2% for the most active site in Walton County (Figure 5). In 2009, sentinel seroconversion rates in 9 out of 19 positive sites exceeded the 18.5% average for the study period (Figure 5).

A total of 7,653 mosquitoes were collected during the course of this study (May-August, 2009), representing 30 different mosquito species. The most abundant species in this region were *Anopheles crucians* Weidemann, *Culiseta melanura* (Coquillett), *Culex erraticus* (Dyar and Knab), and *Culex nigripalpus* Theobald (Figure 6). Greater numbers of *Aedes vexans* (Meigen) and *Aedes infirmatus* Dyar and Knab were collected in tree plantation habitat than other habitat types (Table 4). When normalized by trap night, far more *Ae. vexans* females (80% of the total) were collected in tree plantation habitat than all other habitat types. Similarly, 74% of all *Ae. infirmatus* were collected in tree plantations. In contrast, collections of *Cs. melanura*, the mosquito thought to be the primary enzootic vector of EEEV [85], were relatively evenly distributed across all habitat classes (Table 4). EEEV was detected in 5 separate pools of *Cs. melanura* with a minimum infection rate of 13.24/1000. EEE virus was isolated from 4 of these positive pools. Additionally, the EEE viral genome was detected by RT-PCR in one pool of *Coquillettidia perturbans* (Walker) and one pool of *Psorophora ferox* (von Humboldt). However, virus was not recovered from either of these pools when cultured.

**Discussion**
In an effort to reduce the exposure of EEE to both human and equestrian populations, consideration needs to be taken as to which habitats have an increased risk for disease transmission and how much of that habitat is currently being monitored. Historically, EEEV transmission has been associated with freshwater wetlands, swamps, and marshes [86]. Wetland habitats comprise 23% of Walton County’s land area, and 23% of the total wetland habitat falls within their buffer area surrounding the sentinel sites. This indicates that the sentinel surveillance program in Walton County covers the wetland habitat in proportion to its actual abundance, so that historically at-risk habitats (swamps, marshes) are covered by the activities at their current sentinel sites.

Tree plantation and wetland forested mixed habitats were found to be positively associated with EEEV activity in Walton County. The finding that wetland forest mixed habitat was positively associated with EEEV activity was in keeping with the understanding of the ecology of EEEV developed from studies in the Northeast, where wetland habitats have been shown to be associated with EEEV activity [19]. More surprisingly, this analysis suggested that tree plantation habitat was the habitat classification most significantly positively associated with EEEV activity. Tree plantations primarily consist of forest regeneration areas and coniferous plantations. Florida’s climate allows for rapid growth of several different types of tree species, and due to the productive timber industry, Florida allocates large portions of land for tree plantations. In Walton County, tree plantations were shown to be a positively associated with EEEV transmission. Tree plantations make up 23% of Walton County’s habitat; however, this habitat only comprises 7% of the area currently monitored by sentinel flocks. Within the sentinel sites, the greater the acreage amount of tree plantations
correlated with an increase in sentinel seroconversions to EEEV. Epizootic sites had 3.5 times more acres of tree plantations per site then the negative sites and 2 times more acres then the periodically epizootic sites. One possible reason behind this positive association is that the tree plantations often have a high number of trees per acre [76]. The density and availability of these trees may make the habitat more attractive to nesting and roosting birds. This would increase the potential population and density of reservoir hosts thereby increasing the chance and risk of virus transmission.

We found that vegetated non-forested wetland (primarily treeless hydric savanna) and upland coniferous forests were negatively associated with the transmission of EEEV to sentinel chickens in varying degrees. These habitat types comprise 18% of the county’s total land use and are represented in the sentinel site habitats at 30% of the total area monitored. The reason for the negative association of these wetland types with EEEV transmission is not clear, given the historical connection between wetland habitats and epizootics of EEEV in the Northeasten USA. As wetlands are generally conducive to mosquito breeding, and many wetlands in Florida are protected areas in which insecticide spraying is generally prohibited, mosquito densities in these habitats are generally high. It may be that the negative association may therefore relate more to the availability of competent reservoir hosts than to vector densities. For example, past studies have shown that there tends to be higher production of seed and fruit producing plants, as well as insects, in hardwood swamps as opposed to coniferous swamps [87]. The difference in food resources between wetlands may play a role in habitat preference or foraging areas for potential avian hosts. Similarly the lack of trees in this vegetated wetland may reduce the number of roosting birds, thereby limiting the contact opportunities of vectors with
potential hosts. Regardless of the explanation for this negative association, it is clear that the relationship is not due to an under-representation of these wetland habitats in the buffer area surrounding sentinel sites.

*Culiseta melanura* is typically considered a wetland mosquito; however, our results indicate that in the Florida panhandle this species is not as habitat specific as reported in the northeastern United States [88]. *Culiseta melanura* appeared in all habitat types within this study. Since *Cs. melanura* is considered the primary vector of EEEV, its abundance in a wide range of habitats, especially residential areas, could explain (in part) why there are almost twice as many human cases of EEE in Florida than any other state.

Mosquito species distributions among the different habitats were fairly uniform, except for the tree plantations. In tree plantations, seven times more *Ae. vexans* were collected per trap night than any other habitat type. Four times more *Ae. infirmatus* were also collected in the tree plantations, in relative to other habitats. *Aedes infirmatus* and *Ae. vexans* have been implicated as potential bridge vectors for EEEV [62, 83, 89] and their greater abundance in the tree plantation habitat points towards their possible roles in EEEV transmission. This idea is supported by the seroconversions of chickens during the time of increase in the abundance of these two mosquitoes. Furthermore, their lack of abundance in the wetland habitats may be a factor contributing to the lower transmission rates within those areas. Vector population density is an important driver in EEEV transmission. Periods of EEEV activity frequently coincide with peaks in densities of relevant vector populations. By temporally associating EEEV activity (measured by
chicken seroconversions) with mosquito densities (measured by trapping) it might be possible to implicate potentially important vector species in this process.

The data presented above suggests that increasing monitoring of tree plantation habitat may be a way to increase the efficiency of EEEV surveillance activities in the Florida panhandle. The unique orderly arrangement of trees in tree plantations allows them to be easily recognized through GIS. Tree plantations fall into the industrial forestry category and therefore are not typically environmentally protected, as are many wetlands. Targeting tree plantations for surveillance could improve the protection of both human and horse populations in the Panhandle region of Florida by providing earlier warnings of transmission activity and more effective opportunities for resource management and prevention measures.

References


32. Florida Department of Transportation, *Florida Land Use, Cover And Forms Classification System.* 1999.


Table 1. Habitat and acreage composition of Walton County Florida

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Acres</th>
<th>County Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upland Forest</td>
<td>175,379</td>
<td>26%</td>
</tr>
<tr>
<td>Wetland</td>
<td>157,534</td>
<td>23%</td>
</tr>
<tr>
<td>Tree Plantations</td>
<td>118,831</td>
<td>23%</td>
</tr>
<tr>
<td>Cropland and Pastureland</td>
<td>61,000</td>
<td>9%</td>
</tr>
<tr>
<td>Residential</td>
<td>27,374</td>
<td>4%</td>
</tr>
<tr>
<td>Other (x=24)</td>
<td>118,723</td>
<td>15%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>699,714</strong></td>
<td><strong>100%</strong></td>
</tr>
</tbody>
</table>
Table 2. Results from the Kruskal-Wallis Test between the three classifications (df=2) (P<0.05). Mean hectares of various habitat types by the site classification

<table>
<thead>
<tr>
<th>Land Use Description</th>
<th>Enzootic (mean area [ha])</th>
<th>Periodic (mean area [ha])</th>
<th>Negative (mean area [ha])</th>
<th>H</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cropland and Pastureland</td>
<td>102.3</td>
<td>4.98</td>
<td>161.15</td>
<td>8.57</td>
<td>0.014</td>
</tr>
<tr>
<td>Lakes and Streams</td>
<td>18.62</td>
<td>36.16</td>
<td>1.7</td>
<td>5.54</td>
<td>0.063</td>
</tr>
<tr>
<td>Mixed Rangeland</td>
<td>75.33</td>
<td>76.89</td>
<td>15.17</td>
<td>2.25</td>
<td>0.325</td>
</tr>
<tr>
<td>Residential</td>
<td>157.46</td>
<td>198.1</td>
<td>199.76</td>
<td>1.99</td>
<td>0.369</td>
</tr>
<tr>
<td>Tree Plantations</td>
<td>303.36</td>
<td>89.27</td>
<td>22.69</td>
<td>7.12</td>
<td>0.028</td>
</tr>
<tr>
<td>Upland Coniferous Forest</td>
<td>228.73</td>
<td>317.72</td>
<td>109.64</td>
<td>5.12</td>
<td>0.077</td>
</tr>
<tr>
<td>Upland Hardwood Forest</td>
<td>173.58</td>
<td>69.06</td>
<td>27.2</td>
<td>4.28</td>
<td>0.118</td>
</tr>
<tr>
<td>Vegetated Non-forested Wetland</td>
<td>68.28</td>
<td>179.72</td>
<td>352.02</td>
<td>9.0</td>
<td>0.011</td>
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<tr>
<td>Wetland Coniferous Forest</td>
<td>95.54</td>
<td>175.4</td>
<td>426.82</td>
<td>4.1</td>
<td>0.129</td>
</tr>
<tr>
<td>Wetland Forested Mixed</td>
<td>207.42</td>
<td>84.25</td>
<td>95.06</td>
<td>5.26</td>
<td>0.072</td>
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<tr>
<td>Wetland Hardwood Forest</td>
<td>72.11</td>
<td>23.66</td>
<td>7.12</td>
<td>1.17</td>
<td>0.557</td>
</tr>
</tbody>
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Table 3. Sentinel EEE seroconversion rates per year of the study

<table>
<thead>
<tr>
<th>Year</th>
<th>EEE-Positive Chickens</th>
<th>Total Number of Susceptible Chickens</th>
<th>Seroconversion Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2005</td>
<td>96</td>
<td>348</td>
<td>27.6</td>
</tr>
<tr>
<td>2006</td>
<td>20</td>
<td>250</td>
<td>8.0</td>
</tr>
<tr>
<td>2007</td>
<td>37</td>
<td>109</td>
<td>3.7</td>
</tr>
<tr>
<td>2008</td>
<td>25</td>
<td>254</td>
<td>9.8</td>
</tr>
<tr>
<td>2009</td>
<td>64</td>
<td>342</td>
<td>18.7</td>
</tr>
<tr>
<td>Total</td>
<td>242</td>
<td>1303</td>
<td>18.5</td>
</tr>
</tbody>
</table>
Table 4. Average mosquito collection per trap night per habitat type from May-August 2009

<table>
<thead>
<tr>
<th>Species</th>
<th>Residential</th>
<th>Tree Plantation</th>
<th>Wetland Forest</th>
<th>Wetland Forested Mixed</th>
<th>Upland Forest</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ae. albopictus</em></td>
<td>0.12</td>
<td>0.1428</td>
<td>0.209</td>
<td>1.166</td>
<td>0.059</td>
</tr>
<tr>
<td><em>Ae. infirmatus</em></td>
<td>0.75</td>
<td>13.14</td>
<td>0.175</td>
<td>3.44</td>
<td>0.198</td>
</tr>
<tr>
<td><em>Ae. vexans</em></td>
<td>0.41</td>
<td>14.57</td>
<td>0.2975</td>
<td>2.88</td>
<td>0.112</td>
</tr>
<tr>
<td><em>An. crucians</em></td>
<td>1.08</td>
<td>6.28</td>
<td>0.429</td>
<td>2.5</td>
<td>0.68</td>
</tr>
<tr>
<td><em>Cq. peturbans</em></td>
<td>0.589</td>
<td>2.14</td>
<td>0.517</td>
<td>1.166</td>
<td>0.209</td>
</tr>
<tr>
<td><em>Cs. melanura</em></td>
<td>1.705</td>
<td>0.428</td>
<td>0.824</td>
<td>0.77</td>
<td>0.72</td>
</tr>
<tr>
<td><em>Cx. erraticus</em></td>
<td>0.3</td>
<td>1.28</td>
<td>0.443</td>
<td>3.27</td>
<td>0.424</td>
</tr>
<tr>
<td><em>Cx. nigripalpus</em></td>
<td>0.933</td>
<td>0</td>
<td>0.941</td>
<td>0</td>
<td>0.345</td>
</tr>
<tr>
<td><em>Cx. quinquefasiatus</em></td>
<td>0.187</td>
<td>0.142</td>
<td>0.078</td>
<td>0</td>
<td>0.066</td>
</tr>
<tr>
<td><em>Ps. ferox</em></td>
<td>0.125</td>
<td>1.57</td>
<td>0.117</td>
<td>0</td>
<td>0.101</td>
</tr>
<tr>
<td>Other</td>
<td>0.098</td>
<td>0</td>
<td>0.239</td>
<td>0.833</td>
<td>0.069</td>
</tr>
</tbody>
</table>
Figure 1. Location of Walton County
Figure 2. Study site classifications in Walton County based on sentinel seroconversion activity: Circles = enzootic, triangles = periodically enzootic, and squares = negative sites
Figure 3. Satellite images and habitat classifications of sites in Walton County: Panel (a): Satellite image of an enzootic sentinel site in Walton County. Panel (b): Land use land cover data used to analyze the enzootic sentinel site habitat. Panel (c): A satellite image of a negative sentinel site in Walton County. Panel (d): Land use land cover data used to analyze the negative sentinel site habitat.
Figure 4. Scatter plots with best-fit lines and equation depicting the relationship between habitat contribution: X axis = acres of habitat present. Y axis = EEEV seroconversions in sentinel flocks.
Figure 5. Seroconversion rates by site in 2009. Circles represent enzootic sites and triangles represent periodically enzootic sites. None of the sites classified as negative had any seroconversions in 2009.
Figure 6. Total mosquito collections from May to August 2009, Walton County Florida
CHAPTER 2

Spatial Epidemiology of Eastern Equine Encephalitis in Florida

Note to Reader

This chapter has been previously published (Vander Kelen et al., *Spatial epidemiology of eastern equine encephalitis in Florida*. International Journal of Health Geography, 2012. 11: p. 47.2012) and is utilized with permission from the publisher.

Abstract

Eastern Equine Encephalitis virus (EEEV) is an alphavirus with high pathogenicity in both humans and horses. Florida continues to have the highest occurrence of human cases in the USA, with four fatalities recorded in 2010. Unlike other states, Florida supports year-round EEEV transmission. This research uses GIS to examine spatial patterns of documented horse cases during 2005-2010 in order to understand the relationships between habitat and transmission intensity of EEEV in Florida. Cumulative incidence rates of EEE in horses were calculated for each county. Two cluster analyses were performed using density-based spatial clustering of applications with noise (DBSCAN). The first analysis was based on regional clustering while the second focused on local clustering. Ecological associations of EEEV were examined using compositional analysis and Euclidean distance analysis to determine if the proportion or proximity of certain habitats played a role in transmission. The DBSCAN algorithm identified five distinct regional spatial clusters that contained 360 of the 438 horse cases. The local clustering resulted in 18 separate clusters containing 105
of the 438 cases. Both the compositional analysis and Euclidean distance analysis indicated that the top five habitats positively associated with horse cases were rural residential areas, crop and pastureland, upland hardwood forests, vegetated non-forested wetlands, and tree plantations. This study demonstrates that in Florida tree plantations are a focus for epizootic transmission of EEEV. It appears both the abundance and proximity of tree plantations are factors associated with increased risk of EEE in horses and therefore humans. This association helps to explain why there is are spatially distinct differences in the amount of EEE horse cases across Florida.

**Introduction**

Eastern Equine Encephalitis virus (EEEV) is a highly pathogenic arbovirus endemic to North, Central, and South America. The mortality rate for symptomatic cases of EEE is 35% or more with survivors facing disability from neurological sequelae [16]. From 1964-2010, human cases of EEEV were reported in 20 U.S. states [21], with Florida being the most affected, accounting for 25% of all reported human fatalities to EEE. The enzootic transmission of EEEV is maintained in a mosquito-avian cycle predominantly involving the vector *Culiseta (Climacura) melanura* (Coquillett) and passerine birds [85, 90]. The epizootic cycle of EEEV involving humans and horses involves bridge vectors that are known to feed on both avian and mammalian hosts. Documented and proposed bridge vector species include *Aedes (Aedimorphus) vexans* (Meigen), *Coquillettidia (Coquillettidia) perturbans* (Walker), *Culex (Melanoconion) erraticus* (Dyar and Knab), *Culex (Culex) nigripalpus* Theobald, *Ochlerotatus (Ochlerotatus) canadensis* (Theobald), and *Ochlerotatus (Ochlerotatus) sollicitans* (Coquillett) [1, 84, 91]. Enzootic EEEV transmission has
been associated with hardwood swamp habitats [83] and tree plantations [92]; however, little is known about the ecological associations in the epizootic transmission sites.

EEE is a reportable human and veterinary disease in the United States [93]. In the northeast and south central states, epizootic outbreaks involving humans and horses peak in August and September [24]. In contrast, EEEV transmission in Florida occurs throughout the year, with most human and horse cases occurring in June and July [25]. From 2005-2010, the United States had 1380 horse fatalities from EEE, of which 442 were in Florida (32%) [94]. Despite the availability of an effective equine EEEV vaccine, Florida averages 70 EEEV equine case fatalities per year. Currently there is no approved vaccine for humans or effective medical treatment for those infected with the virus. Prevention strategies to protect the human population from EEE thus rely primarily upon case detection and vector control.

In previous studies, spatial methods were used to associate particular habitats with seroconversions of sentinel chickens to EEEV in Walton County, Florida [92]. Because EEEV is maintained in an enzootic cycle involving passerine birds as the vertebrate reservoir and chicken sentinels attract ornithophilic mosquito species that serve as the enzootic vectors for the virus, this study primarily assessed habitats associated with the enzootic cycle. Through the use of spatial epidemiology, this research aims to improve our understanding of the ecology of EEEV in Florida by examining the spatial distribution and habitat associations of documented horse EEE fatalities through examining habitats associated with the epizootic cycle in which mammals are exposed to the virus.
Spatial epidemiology is the study of the geographical variation in disease risk or incidence [95]. As a growing field, spatial epidemiology provides new insights into arbovirus transmission as it pertains to environmental interactions. Geographic Information Systems (GIS) and remote sensing are just a few of the tools used to measure spatial variation in disease risk [51-55]. In terms of arthropod-borne diseases, GIS has been employed to analyze environmental factors associated with Lyme borreliosis [96, 97], tick-borne encephalitis [98], West Nile virus [57, 59, 60, 71], Dengue virus [99, 100], and Eastern Equine Encephalitis virus [19, 92]. Spatial clustering is a GIS technique routinely utilized to explore patterns of disease transmission. Identifying the geographical location and distribution of disease allows researchers the opportunity to analyze the potential local or regional drivers of disease transmission. Research has shown that areas with spatial clustering of vectors and hosts may increase the risk of disease transmission [101]. Spatial clustering methods have also successfully been used to detect high risk areas for West Nile virus [51, 102] and Ross River virus [103]. This study applies clustering and other spatial epidemiological techniques using GIS to understand the spatial variation in horse cases of EEEV in Florida. The main goals of the research were to: (1) identify counties with the highest incidence rates of EEE in horses, (2) explore regional and local clusters of EEE horse fatalities, and (3) determine habitats associated with EEEV in horses, in terms of both abundance and spatial proximity.

Methods

The state of Florida covers an area of about 170,304 km². It is the only state with both subtropical and tropical regions. Florida is made up of five major land cover classes which collectively account for 94% of state’s habitat. These include wetlands (27%),
upland forests (24%), agriculture (19%), urban (13%), and water (11%). Due to Florida’s high water tables, wetland areas tend to be fragmented and intermixed between other land cover classes, creating a complex mixed ecosystem [104].

GIS layers documenting Florida habitats were obtained from the state's five Water Management Districts. The Florida Department of Environmental Protection's Bureau of Watershed Restoration developed these land use and land cover maps using the Digital Ortho Quarter Quad Aerial Imagery program Color Infrared and True Color photography [75]. The schema of habitat classification descriptions for the land use-land cover encompassed four different levels, with Level 1 being the most basic and Level 4 the most specific [76]. In this study, ecological habitats were characterized using Level 2 land cover usage classifications. Level 2 descriptions were selected because they differentiated between various wetland types, as well as different residential features. The 42 sub-classifications found in the Level 2 categories were aggregated to 14 classes for use in this study: (1) Low Residential, (2) Crop and Pastureland, (3) Upland Hardwood Forest, (4) Vegetated Non-forested Wetland, (5) Tree Plantations, (6) Wetland Mixed Forest, (7) Upland Coniferous Forest, (8) Wetland Coniferous Forest, (9) Medium and High Density Residential, (10) Urban, (11) Water, (12) Wetland Hardwood Forest, (13) Shrub and Brushland, (14) Mining. The selected land use classifications were chosen based on their overall dominance and suspected habitat importance to equine populations and mosquito vectors associated with EEEV. For instance, certain water classes were combined (lakes, reservoirs, etc), as were high and medium density residential classes. Tree crops and tree nurseries, low in abundance, were combined in the tree plantations class. The remaining classes were placed into an urban category (e.g. large paved areas,
buildings, and airports). Coastal habitats were excluded from the study, since EEEV is only transmitted by freshwater mosquitoes.

Florida had a total of 442 reported horse cases of EEE from 2005-2010 [105]. Case locations were georeferenced using GPS coordinates provided by the Florida Department of Health. Four cases in this database were excluded due to incomplete or missing coordinates, leaving 438 cases that were included in the analysis. Between 2005-2010, 54 out of 67 counties reported the occurrence of at least one horse case of EEE. Of the 10 counties with no horse cases of EEE, 8 were coastal counties. To establish the incidence of EEE horse cases, total equine populations were acquired for each county [106]. Cumulative disease incidence was then calculated by dividing the number of horse cases per county from 2005-2010 by the 2007 horse census population totals. The Spearman’s rank correlation coefficient was used to test if there was a relationship between the number of cases and the total population of horses in each county.

To characterize the spatial pattern of EEE horse cases in Florida, the Density-Based Spatial Clustering of Applications with Noise (DBSCAN) technique was employed. DBSCAN is one of the most widely applied spatial clustering methods, since it can detect clusters of complex shapes and can operate at different spatial scales [107]. The algorithm works by moving point to point based on the (x,y) coordinates of each case and calculates the density-reachability and point connectivity between cases; these values are then used to either assign points to particular cluster or designate them as statistical noise [107]. DBSCAN requires the user to specify two input parameters: the minimum number of points used to define a cluster (minPoints) and the neighborhood distance for defining clusters (epsilon). Two spatial clustering analyses were conducted using
different DBSCAN parameters to examine both the regional and local clustering of cases. The parameters used to verify regional case clusters were a minimum of eight points and an epsilon distance of 25,000 meters for connectivity. Local clustering parameters were a minimum of four points and 6,000 meters for connectivity. Cases contributing to each clusters were examined by year to determine temporal disease patterns.

A compositional analysis of habitat use, which is widely used in ecology to identify habitat use by wildlife [108-111], was conducted to rank which habitat types were most associated with cases of EEE in terms of proportional abundance [112]. A total of 14 aggregated classes, including; (1) Low Residential, (2) Crop and Pastureland, (3) Upland Hardwood Forest, (4) Vegetated Non-forested Wetland, (5) Tree Plantations, (6) Wetland Mixed Forest, (7) Upland Coniferous Forest, (8) Wetland Coniferous Forest, (9) Medium and High Density Residential, (10) Urban, (11) Water, (12) Wetland Hardwood Forest, (13) Shrub and Brushland, (14) Mining were used in the analysis. Habitats immediately neighboring EEE horse cases were compared to habitats in the surrounding landscape. Spatial scales ranging from 1-2km are commonly utilized to determine the spatial epidemiology of arthropod diseases [19, 59, 113]. The 1.5km distance was chosen because many of the bridge-vector mosquito flight ranges fall within this buffer range [78, 114, 115] and it has been successfully used in previous studies to determine landscape associations of enzootic EEEV activity in Florida [92]. Habitat proportions for each case were calculated from a 1.5km buffer around each individual site. Available habitats were calculated by considering the total habitat composition in the surrounding county [116, 117]. The results of the analysis were summarized using a
ranking matrix, which identified which habitats are proportionally most associated with EEE as compared to habitats available in the surrounding landscape.

A Euclidean distance analysis [118, 119] was conducted to detect the proximity of each horse case of EEE to each of the 14 habitat classifications used in this study. Each individual horse case was used as a source point to calculate the distance (meters) to the nearest location in the landscape of each habitat type. These results were also used to compare the observed horse case distances to similar distances for all other locations in the surrounding landscape. Here, the surrounding landscapes were defined based on four ecoregions: Panhandle, North, Central, and South (Figure 7). The purpose of the division was to account for any regional ecological differences, so that each horse case is compared to other areas with similar habitat and landscape configurations. For each region, the median distance from horse cases to each habitat type was compared to the regional median using a Wilcoxon test. A nonparametric test was used because the distances for horse cases were not normally distributed. A Bonferroni correction was used in testing for statistical significance. The purpose of the comparison is to identify if horse cases are located closer to particular habitat types than would be expected for each region.

Results

Florida contained a total of 120,614 horses according to the 2007 equine census data [106], with all but two counties having horses. The highest density of horses occurred in the Northern region of Florida. County based culmulative incidence rates of EEE for 2005-2010 varied across the state. The average incidence rate per county per year was 1 case of EEE per 1,000 horses. Fourteen counties had culmulative incidence
rates of 2 cases per 1,000 horses per year or higher with 10 of the 14 being in the Northern region (Figure 8). Washington county, located in the Panhandle region, had the highest incidence rate of EEE cases at 12 per 1,000 horses per year. The area with the lowest incidence rates was the Southern region, despite the fact that 4 of the 7 counties reported horse cases. Ten counties had no horse cases during 2005-2010, of which 8 were coastal counties. The results of the Spearman’s rank correlation coefficient showed that there was no significant relationship between the number of cases and the total population of horses in each county ($\rho = 0.24$, $p = 0.06$). Washington County had the highest incidence of disease while having one of the lowest county horse populations, while Marion county had a large population of horses and a low incidence rate.

DBSCAN identified five regional EEE case clusters across Florida during 2005-2010 (Figure 9). Case clusters included 360 out of the 438 cases with the remaining 78 cases being identified as statistical noise. The largest clusters were Cluster 1 in North Florida (145 cases) and Cluster 5 (66 cases) in the Central Region (Table 5). Cluster 1 had contributing cases every year, averaging 24 cases per year with a maximum of 51 in 2005 and a minimum of 4 in 2007. Cluster 5 had an average of 11 cases per year with a maximum of 25 in 2005 and a minimum of 2 in 2006 and 2007. The smallest Cluster was cluster 4 which had 33 cases over all, with no contributing cases in 2007. The most productive year for EEE cases in the regional clusters was 2005, with 120 cases included.

DBSCAN identified 18 local clusters in 17 different counties throughout Florida. A total of 105 (24%) of all cases were within the local clusters (Table 6). Ten of the 18 clusters were located in the North region of Florida, with only one cluster found in the South. Five of the seventeen counties (Holmes, Washington, Marion, Volusia, and
Osceola) had two local clusters within their boundaries. None of the local clusters had consistent yearly case contributions for all six years. However, the average cluster had cases in three of the six years. Cluster 7 had 11 contributing cases out of the 105 and had activity in four of the six years. Clusters 6 and 7 had a combined sum of 20 cases representing 19% of the total cases within the local clusters; both of these clusters were located in Washington County. The most productive years for EEE cases in the local clusters were 2005 and 2008, both years reporting 30 cases (Table 6).

The predominant habitat in terms of abundance around the cases was cropland pastureland, comprising 25% of the area within the buffers. Tree plantations were the second most abundant feature (at 15%) with low density residential land shortly behind at 12%. Wetland coniferous forest, wetland hardwood forest, vegetated non-forested wetland and wetland forested mixed collectively comprised 18% of the habitat within the buffers (Table ). The habitat compositional analysis revealed that five land cover classes were proportionally more abundant in the buffer area of the horse cases than in the surrounding landscape. The top five classes, in rank order, included: (1) low density residential, (2) crop and pastureland, (3) upland hardwood forest, (4) vegetated non-forested wetlands, and (5) tree plantations (Table 8). Six categories—urban, water, medium density residential, wetland hardwood forest, shrub and brushland, and mining—were less abundant in the buffers than in the surrounding landscape.

Euclidean distance analysis was applied in order to measure the spatial proximity of different habitats to EEE horse cases. Since the resulting distances were not normally distributed, they were summarized by medians rather than means (Table 9). EEE horse cases were on average closest to low density residential land (30-79m) and crop and
pastureland (90-120m). Other habitats showed slight differences in proximity rankings, with upland hardwood forest, vegetated nonforested wetland, and tree plantations tending to be the next nearest habitats. Cases were on average 152-1034 m from upland hardwood forest, significantly closer than expected for all four regions. The median distances for vegetated nonforested wetland were 346-681 m, although these distances were either insignificant or significantly farther than expected based on the configuration of the surrounding landscapes. Cases averaged 248-694 m from tree plantations, significantly closer than expected in all but the North region. Horse cases were located farther from to the other habitat types, with various wetland types tending to be the next proximal, although the distances vary widely by region.

Discussion

Regional clustering of EEE horse cases highlighted the spatial differences of EEE transmission in Florida. The regional case clusters included 360 out of the 438 cases, which illustrates a spatial component in the transmission of EEEV to horses. The northern region accounted for 46% of the total horse cases from 2005-2010, as well as exhibited the highest incidence rates. In low transmission years, case clustering mainly takes place in the northern region of Florida. The regional clustering focuses on the inland counties reinforcing the lack of cases in the coastal counties, which are more dominated by saltwater marshes where EEEV is not endemic.

The local clustering highlights the focality of EEE transmission to horses and the density of cases within specific counties. The spatial location of the local clusters varied across the state. The most densely clustered area was in the panhandle region in Washington and Holmes counties. This area had four localized clusters, accounting for 29
out of the possible 106 cases and had cluster-contributing cases in all years except 2006. Local clusters were present in 18 of the 67 counties with 4 counties having more than one cluster. The northern region contained 10 of the 18 local clusters, which implies a strong focal nidus of transmission in this region. Results from the DBSCAN clustering method supports previous findings in which EEEV amplification was related to localized ecological conditions [65].

The finding that cropland and pastureland were the most abundant habitats surrounding equine cases of EEE was not surprising since that is where horses are typically found. The same can be said for low density residential areas, since it is the rural communities that have enough land area to support horse populations. Tree plantations, comprising 15% of the area around EEE cases, were found to be the next most abundant habitat associated with equine cases. The median distance of tree plantations from horse cases revealed that 50% of the cases fell within 470 m—significantly closer than expected for all regions in the state—suggesting that the proximity of tree plantations surrounding EEE cases may be an important factor in EEEV transmission to horses. The compositional analysis confirmed that tree plantations were over-represented in the EEE case buffers compared to its availability in the surrounding area.

Tree plantations seem to be an important ecological factor in EEEV transmission to horses in Florida. Previous studies in Walton County Florida have shown that tree plantations were associated with enzootic EEEV transmission [92]. The enzootic cycle of EEEV transmission involves avian hosts and the vector *Cs. melanura*; while the epizootic cycle involves equines and humans and various possible bridge vectors. The association
of tree plantations with the risk of EEEV transmission in both sentinel chickens and horses suggests that tree plantations might harbor enzootic foci from which EEEV emerges into its epizootic cycle. One explanation is that the tree plantations often have a higher number of trees per hectare compared to other forest types [76]. The density and availability of these trees may make the habitat more attractive to nesting and roosting birds, and thereby increasing the intensity of both enzootic and epizootic EEEV activity. The tree density may also provide suitable sheltering locations for various mosquito species. The location of crop and pasturelands next to the tree plantations also might provide an edge effect, allowing for a greater concentration of both vector and avian populations within the horse habitats [120]. A concurrent explanation is that the tree plantation habitats often rest on poorly drained soil. The poor soil drainage could result in the inundation of the area, thereby creating temporary wetland conditions in close proximity to horses. Previous studies have shown that hydrologic conditions due to variations in temperature and rainfall can influence arbovirus vectors and hosts resulting in increased risk for dispersal into the surrounding areas [121, 122].

Upland hardwood forests were found to be both significantly closer to horse cases then random (p<0.003) in all four regions. This suggests that the having upland hardwood forests located near areas with horses might be associated with a greater risk for EEEV transmission. The close proximity of cases to upland hardwood forests suggests that this may be a viable habitat for an EEEV vector. The primary vector of EEEV, Cx. melanura, is a hardwood swamp mosquito [85]. Although the upland hardwood forest is not classified as a wetland, it does contain mesic communities which are considered moderately moist sites [76]. Furthermore, the dense canopy cover reduces air circulation
causing increased humidity within this land cover [123]. These conditions may provide adequate breeding sites for *Cs. melanura* thereby increasing the vector’s distribution among different habitats. Previous research has shown that in Florida *Cs. melanura* is evenly distributed across all habitat types, including hardwood forests [92].

Wetland hardwood forests, the habitat most often associated with the EEEV vector *Cs. melanura* in the northeast United States [85, 124], ranked 12th out of 14 in the compositional analysis of habitat use (Table 8). Furthermore, the median distance from cases was 696m compared to the state median of 654m. These results may indicate that wetland hardwood forests do not play as critical a role in the epizootic EEEV transmission cycle in Florida as it appears to play in the Northeastern states. This is supported by previous research of habitat associations with enzootic transmission which showed there was no association of EEEV transmission with wetland hardwood forests [92]. This may be the result of the vector *Cs. melanura* not being as confined to a specific habitat type in Florida [92]. Further studies need to be conducted to determine the affect wetland hardwood forests have on EEE transmission in Florida.

Finally, while this study implicates several habitats associated with EEE horse fatalities in Florida, there are other factors not analyzed that play a role in EEEV transmission and could explain, at least in part, the spatial patterns observed. Such a factor is the availability of a vaccine against EEEV for horses which requires semiannual boosters to ensure protection from EEE. However, vaccine usage is not tracked, and this adds a potentially confounding variable to the study if horse vaccination rates vary across the state. For example, if vaccination rates are lower in the Northern region, this could explain why there is such a high incidence of EEE horse fatalities despite low population
densities. If future studies are able to explore vaccination rates, then researchers can better understand the role of habitat in EEEV transmission.

Overall, the results of this spatial epidemiological study have demonstrated that EEE horse fatalities cluster in farmlands and rural residential lands that are located near wetlands and tree plantations. Identifying locations in Florida that exhibit these types of habitat configurations could ultimately be used to prevent EEEV transmission by targeting vector control measures in the highest risk areas. Future work might explore GIS-based models to predict EEEV transmission based on the results of this work. Furthermore, these findings are relevant to other locales with endemic EEEV that also have subtropical and tropical climates. For example, EEEV is endemic to both Central and South America and have endured epizootic outbreaks within their equine populations [125, 126]. Despite human cases of EEE being quite low in South America, epizootic outbreaks have been known to effect thousands of horses [127]. By identifying high risk areas through habitat associations, targeted surveillance and prevention methods could be used to limit the impact EEEV has within the at risk populations of these countries, as well. Additionally, the approach used to identify spatial patterns and habitat associations of horse fatalities can be used to guide similar studies of other diseases.

In terms of Florida, specifically, this research highlights the potential importance of tree plantations in EEEV transmission. Tree plantations have been previously shown to be a habitat associated with an increased risk of enzootic EEEV transmission [92]. This study demonstrates that in Florida tree plantations are also a focus for epizootic transmission of EEEV. It appears both the abundance and proximity of tree plantations are factors associated with increased risk of EEE in horses and therefore humans. This
association helps to explain why there is a spatially distinct difference in the amount of EEE horse cases across Florida. Tree plantations are scarce in southern Florida and despite having similar horse populations as the panhandle area, disease incidence is much lower. This study also associates upland hardwood forests with EEEV transmission. Again, both abundance and proximity play a role in increasing the risk of EEEV transmission to horses and humans. The focality of transmission was also highlighted in the local case cluster analysis. It is important to determine the ecological risk factors for EEEV transmission in Florida in order to reduce the number of human and horse cases. Furthermore, understanding the ecology of this disease will help to identify at risk areas, thereby providing better opportunities for vector control. By focusing on high risk habitats, prevention methods can be used to reduce the amount of disease transmission, resulting in better protection for both the equine and human populations in Florida and other areas where EEEV is endemic.
References


76. Florida Department of Transportation, *Florida Land Use, Cover And Forms Classification System*. 1999.


93. CDC, 2012 Case Definitions: Nationally Notifiable Conditions Infectious and Non-Infectious Case. 2012: Atlanta, GA.


Table 5  Regional clusters with cases by year and cluster

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Table 7. Proportions of habitat types within the 1.5km buffer area of EEE horse cases in Florida

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### Table 8. Habitat Compositional Analysis

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<td>14</td>
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</tbody>
</table>

Simplified ranking matrices with 14 land classifications ranked in order of proportional habitat use between horse cases and the surrounding county (+ preference, - avoidance, a triple sign represents significant deviation from random at P < 0.05). LR=low density residential, CP=crop and pastureland, UHF=upland hardwood forest, VNFW=vegetated non-forested wetland, TP=tree plantations, WFM=wetland forested mixed, UCF=upland coniferous forest, WCF=wetland coniferous forest, MR=medium density residential, WHF=wetland hardwood forest, and SB=shrub and brush land.
Table 9. Euclidean Distances from habitats to cases based on ecological regions

<table>
<thead>
<tr>
<th>Habitat</th>
<th>Central</th>
<th>North</th>
<th>Panhandle</th>
<th>South</th>
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<tr>
<td></td>
<td>Horse</td>
<td>Region</td>
<td>Horse</td>
<td>Region</td>
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<td>LR</td>
<td>78.5</td>
<td>1874</td>
<td>&lt;0.001</td>
<td>60</td>
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<tr>
<td>CP</td>
<td>114</td>
<td>150</td>
<td>0.65</td>
<td>94</td>
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<tr>
<td>UHF</td>
<td>516.5</td>
<td>834</td>
<td>0.003</td>
<td>182</td>
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<tr>
<td>VNFW</td>
<td>346.5</td>
<td>240</td>
<td>&lt;0.001</td>
<td>408</td>
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<td>TP</td>
<td>693.5</td>
<td>1106</td>
<td>&lt;0.001</td>
<td>258</td>
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<tr>
<td>WFM</td>
<td>868</td>
<td>1874</td>
<td>&lt;0.001</td>
<td>569</td>
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<td>UCF</td>
<td>744.5</td>
<td>1120</td>
<td>0.046</td>
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<td>WCF</td>
<td>709.5</td>
<td>1574</td>
<td>0.001</td>
<td>715</td>
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<td>Urban</td>
<td>705.5</td>
<td>1449</td>
<td>&lt;0.001</td>
<td>751</td>
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<tr>
<td>Water</td>
<td>362.5</td>
<td>550</td>
<td>0.002</td>
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<td>MR</td>
<td>1383</td>
<td>3156</td>
<td>0.001</td>
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<td>WHF</td>
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<td>0.778</td>
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<td>Mining</td>
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<td>2694</td>
<td>0.326</td>
<td>2057</td>
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</table>

Median Euclidean distances in meters for the four regions in Florida. Results from the Wilcoxon test with the p-value being significant at the 0.0036 after the Bonferroni correction. Horse = median (meters) from horse cases to habitat type. Region = median (meters) from random possible points to habitat type. LR=low density residential, CP=crop and pastureland, UHF=upland hardwood forest, VNFW=vegetated non-forested wetland, TP=tree plantations, WFM=wetland forested mixed, UCF=upland coniferous forest, WCF=wetland coniferous forest, MR=medium density residential, WHF=wetland hardwood forest, and SB=shrub and brush land.
Figure 7. Florida ecological regions. The four ecological regions of Florida in this study include panhandle, north, central, and south.
Figure 8. Cumulative incidence rates per county per year. A) County based cumulative incidence from 2005-2010 per 1,000 horses per year normalized by population. B) County based horse populations from 2007 Census
Figure 9. Density-Based Spatial Clustering of Applications with Noise for EEE cases in Florida from 2005-2010. A) Regional clustering with 5 clusters. B) Local clustering with 18 clusters.
CONCLUSION

The habitat associations of EEEV transmission in Walton County Florida showed that both tree plantations and mixed wetland forests were positively correlated with high rates of EEEV transmission. Furthermore, the enzootic vector *Cs. melanura* was found to be evenly distributed across all available habitat types. Vegetated non-forested wetland (primarily treeless hydric savanna) and upland coniferous forests were negatively associated with the transmission of EEEV to sentinel chickens in varying degrees.

The spatial analysis of horse cases from 2005-2010 also showed tree plantations had a positive association with EEEV transmission rates. The compositional analysis and the Euclidean distance analysis both highlighted that tree plantation abundance and proximity may be indicators for increased EEEV risk. Overall, the research in these studies provides evidence of ecological associations for EEEV transmission in Florida that hasn’t been previously analyzed. Furthermore, these studies provide the groundwork for better understanding of why there is a disproportionate number of horse and human cases of EEEV in Florida than in any other state.

The next step in determining the ecological associations of EEEV in Florida would be to develop an EEEV transmission risk model. Due to the complexity of the EEEV transmission cycle the best method for developing a risk model would be to create a suitability index (SI) model for the disease. SI models are routinely used in GIS to predict probable locations for animal species or other phenomena [128-130]. Typically,
they combine spatial and attribute factors into a mathematical index that rates the suitability of each location on a continuous scale from 0.0 (completely unsuitable) to 1.0 (optimally suitable). In the case of disease, SI models can be used to develop risk maps that similarly evaluate locations on a continuous risk scale of 0.0 (no risk) to 1.0 (highest risk).

There are two different types of possible input data the SI model can use. Categorical data can be used by classifying discrete values based on relative suitability. A continuous scale from 0 to 1 is used to represent the level of suitability the measured variable accounts for. The value of 0 is considered completely unsuitable, 1 is optimal habitat, and all values in between represent varying degrees of suitability. The other type of input data would be to assign values based on a continuous scale according to a linear function. This would be done by assigning a cell value based off of percentages or proportions.

A suitability model for EEEV would focus on predicting areas of highest risk for disease in either humans or horses [54, 131]. A proximity-based approach using GIS that evaluates risk on the basis of habitat conditions as well as the landscape configuration around both sentinel sites and previous horse cases could be an important factor in measuring suitability [132-134]. Using GIS, each grid cell (30x30m resolution) in a raster data format would receive a rating for its potential to support EEEV transmission. Potential SI model variables could include: (1) local environmental factors at the grid cell level such as habitat type, soil type, rainfall or climate, elevation, vegetation health, density of human or horse populations; (2) landscape level factors within a focal neighborhood of each grid cell; and (3) measured or predicted abundance of vector and
host species within a focal neighborhood of each cell. Within each grid cell risk index values would be computed based on observed relationships between EEEV and the supporting factors. To accomplish this, individual risk indices would need to be developed for each variable (freshwater wetlands, tree plantations, rural residential, and cropland) found associated with EEEV transmission. Individual risk values would then range from 0.0 to 1.0 and values would be assigned based on specified discrete or continuous mathematical functions, depending on the observed relationship between the factor and risk. Distance from freshwater wetlands to rural residential or cropland areas would need to be calculated. Any residential or cropland areas that fall within 1.5 km of a freshwater wetland would receive correspondingly higher risk values than those that are located further then 1.5 km. Then an overall risk index would need to be created by using the Analytic Hierarchy Process (AHP) to combine individual risk indices into a mathematical formula that rates EEEV transmission risk on a scale from 0.0 to 1.0. The formulation of the EEEV transmission risk model would depend on the relative contribution of each individual factor to overall risk.

The model could then be validated using archived and new horse case data that were not included in the previous studies [135, 136]. Both true horse cases and random generated points would be used to run the model and assess its prediction accuracy. The goal of the risk model would be to accurately predict positive sites for EEEV horse cases; positive case sites should receive high-risk values (>0.7 for instance), while negative sites should receive lower values.

Ideally, the generated risk model would produce a data layer that both accurately predicts equine cases of EEEV and limits the amount of false positives. The predictive
risk model could then be used to determine which areas have the greatest risk for EEE in equine and human populations. The data gathered from the risk model could then be shared with local County mosquito control district to help improve the efforts of their control measures thereby potentially reducing the risk of disease transmission to humans and horses in Florida. Each mosquito control district would be able to have a working model based on their individual county parameters which would allow them to prioritize their already existing surveillance areas and locate new high risk locations. Once high risk EEEV locations are selected based on the model results, early intervention techniques could be implemented to reduce early season transmission. Interrupting early season transmission may reduce or prevent outbreaks later in the summer when mosquito densities peak. This could be validated by continuing to monitor existing chicken sentinels for seroconversions and comparing rates temporally from previous years to post intervention years. Improving surveillance methods, specifically interrupting early season transmission can save counties on costly late season interventions while simultaneously reducing risk to humans and horses.

References


APPENDIX

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