

Host Feeding Patterns of Potential Vectors of Eastern Equine Encephalitis Virus at an Epizootic Focus in Tennessee

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Abstract. In 2006, 2,817 blood-fed mosquitoes were collected from the site of a 2005 eastern equine encephalitis outbreak in Chester County, TN. Using a polymerase chain reaction–based assay, 264 vertebrate hosts were identified from seven mosquito species. *Culex erraticus* and *Cx. nigripalpus* fed on a diversity of mammalian, avian, and reptilian hosts, whereas *Anopheles quadrimaculatus* and *An. punctipennis* were predominantly mammalophilic. Overall, 27% of *Cx. nigripalpus*, 16% of *Cx. erraticus*, and 7% of *An. quadrimaculatus* blood meals were acquired from avian hosts. No avian-derived blood meals were identified from *An. punctipennis*. The house finch, Carolina wren, and mourning dove were the most commonly identified avian host species. By incorporating this study with flight range, vector competence, and virus field isolation data, we assessed certain aspects of the enzootic and epizootic vectorial capacity of the mosquito species present at this outbreak site.

INTRODUCTION

Eastern equine encephalitis virus (EEEV) is a virulent arbovirus maintained in an enzootic cycle between local avian fauna and ornithophilic mosquitoes in coastal regions of the eastern United States. The virus is severe in horses, where the mortality rate approaches 90%.¹ *Culiseta melanura* is the principal mosquito involved in perpetuating the enzootic cycle.² Although it has been documented to feed on mammals,^{3–5} transmission of EEEV from the avian reservoir to peripheral mammals, namely horses and humans, is most likely accomplished by catholic feeding bridge vector species such as *Aedes sollicitans* and *Coquillettidia perturbans*.^{6,7}

EEEV has been reported at inland sites since the late 1980s,⁸ and equine EEE outbreaks have occurred in Tennessee since 2002 (Tennessee Department of Agriculture data). In 2005, a large equine EEE outbreak occurred in Chester County, TN, where eight horses died of EEE-like symptoms with two laboratory-confirmed cases. A CDC light trap survey conducted at the outbreak site during the summer of 2006 collected 13,955 specimens. *Culex erraticus* (36%) was the most abundant species identified, followed by *Cx. pipiens/restuans* (14%), *Cq. perturbans* (15%), and *Anopheles quadrimaculatus* (11%). *Cs. melanura* comprised 0.7% of the population. Subsequent testing for EEEV did not identify any positive samples from > 2,000 pools, including those of *Cs. melanura* (Mukherjee and others, unpublished data). Furthermore, the typical habitat of *Cs. melanura* was absent at the outbreak site.

Similar patterns of high *Cx. erraticus* and low *Cs. melanura* populations with circulating EEEV have been identified in southeastern swamps of Mississippi and Alabama.^{9,10} To explain this phenomenon, Cupp and others¹¹ hypothesized that *Cs. melanura* has been gradually replaced by *Cx. erraticus* after drastic habitat changes caused by deforestation. The minimum EEEV infection rate in *Cs. melanura* has been high in Alabama regardless of population size, suggesting a continuing role in transmission,⁹ whereas rare virus isolations and

potential low viral activity in Tennessee may suggest deviations from the traditional EEEV transmission system.

Little is known about the transmission dynamics at outbreak foci in Tennessee. Only one other study has examined feeding patterns of mosquitoes in Tennessee, and it focused on peri-urban Memphis, Shelby County, rather than a recognized EEE epicenter.⁵ This study is important because of the large number of farms with unvaccinated horses in rural areas of western Tennessee, and it is increasingly important for public health as urban expansion into sylvatic and rural areas progresses. Enhanced knowledge of the ecologic factors involved in EEE outbreaks will aid in preventing and managing future epizootics. In this study, we seek to elucidate the roles of potential vectors of EEEV at an epizootic focus in Tennessee through molecular bloodmeal analysis.

MATERIALS AND METHODS

Mosquito collections. Blood-fed mosquitoes were collected from eight resting boxes two to three times per week from the first week of June through the last week of September 2006 at three different sites surrounding the epicenter of a 2005 equine EEE outbreak in Chester County, TN. Blood-fed mosquitoes were aspirated from resting boxes, frozen with dry ice, and transported to the Tennessee Department of Health (TDH) Vector-Borne Disease Laboratory where they were stored at –80°C.

Study sites. Site A (35°36.144' N, 88°33.603' W) was an early successional bottomland hardwood forest and full sun emergent marsh heavily impacted by beaver activities. Most over-story trees in the emergent marsh had been killed by beaver girdling or by longer than usual inundation periods from beaver damming. This site was adjacent to a stable where one horse died of EEE-like symptoms during the 2005 outbreak. Horses were present at this stable during the 2006 study.

Site B (35°35.987' N, 88°33.142' W) was a mosaic of wetland types—forested wetland, emergent marsh, and early successional shrub-forest wetland. The emergent wetland, located immediately north of an old railroad bed, contained a man-made impoundment used for duck hunting.

Site C (35°34.678' N, 88°34.031' W) was a disturbed bottomland hardwood forest that was sometimes temporarily

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inundated by the Middle Fork of the Forked Deer River and its adjacent, unnamed tributary. The two laboratory-confirmed EEE horse cases occurred at Site C.

Mosquito identification. Mosquitoes were identified by morphology using a chill table and were stored at -80°C until further testing. Individual *Cx. restuans* and *Cx. pipiens* mosquitoes were distinguished using previously described polymerase chain reaction (PCR) assays.^{12,13}

Isolation of DNA from blood-fed mosquitoes. Abdomens were separated on a chill table using a fresh microscope slide for each sample and flame-sterilizing forceps between samples to prevent cross-contamination. DNA was isolated from mosquito abdomens homogenized in DNAzol-BD (Molecular Research Center, Cincinnati, OH), according to Molaei and others.⁴ Isolated DNA was stored at -80°C until further testing.

Bloodmeal analysis. Extracted DNA served as templates in a PCR to amplify portions of the *cytochrome b* gene. Each mosquito sample was tested against avian and mammalian primers, as previously described.⁴ Because of sequence homology among classes, these primer sets also amplify reptilian DNA. All PCRs were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA).

Positive PCR products were treated with Exosap-It (USB, Cleveland, OH) for purification and either sequenced at the TDH Laboratory Services (Nashville, TN) with a 3130 \times 1 genetic sequencer (Applied Biosystems) using BigDye Terminator (Applied Biosystems) or sent to the MWG sequencing facility (MWG Biotech, Huntsville, AL) for analysis. Sequences were entered into the NCBI BLAST database for identification. Only matches with at least 96% identity were accepted.

RESULTS

During the summer of 2006, 2,817 blood-fed females representing 4 genera and 10 species were collected using resting boxes. The population was comprised of 2,498 (88.6%) *An. quadrimaculatus*, 165 (5.8%) *Cx. erraticus*, 65 (2.3%) *An. punctipennis*, and 63 (2.2%) *Cx. nigripalpus*; other mosquitoes collected include 9 (0.3%) *Cx. territans*, 8 (0.28%) *Cx. pipiens*, 4 (0.1%) *Cs. melanura*, 2 (0.07%) *An. crucians*, 2 (0.07%) *Culex* spp. mosquitoes that could not be identified using molecular assays, and 1 (0.04%) *Cq. perturbans*.

We tested a sampling of 203 *An. quadrimaculatus* mosquitoes to represent each month and collection site equally and 291 (91%) of the remaining species for bloodmeal identification. Vertebrate host species were successfully identified to class in 301 of 494 (61%) specimens tested. Of those 301, 264 (88%) were positively identified to species.

The majority of identified bloodmeals were derived from large mammals such as horses and white-tailed deer (Table 1). *Cx. erraticus* and *An. quadrimaculatus* also fed on a diversity of small mammalian species, and a human-derived bloodmeal was identified from *Cx. nigripalpus*. Reptilian blood meals were identified from *Cx. erraticus* (7% of total blood meals) and *Cx. nigripalpus* (10%). Two horse-acquired and one white-tailed deer-acquired bloodmeals were identified from *An. crucians* and *Cx. pipiens*, respectively.

Avian bloodmeals were identified from four mosquito species (Table 1). Only *Cs. melanura* was considered ornithophilic. In contrast, *Cx. nigripalpus*, *Cx. erraticus*, and *An. quadrimaculatus* acquired 27%, 16%, and 7% of total bloodmeals from

avian hosts, respectively. The most commonly identified avian hosts were the house finch (22% of all avian bloodmeals), the Carolina wren (16%), and the mourning dove (16%).

DISCUSSION

With the exception of *Cs. melanura*, all mosquitoes from which vertebrate hosts were identified fed predominantly on mammals. Mosquito affinity for white-tailed deer has been observed in similar studies, where it was attributed to white-tailed deer size, abundance, and mosquito tolerance.^{4,5,14,15} It has been postulated that the abundance of white-tailed deer may dilute disease transmission by diverting catholic-feeding mosquitoes away from avian-amplifying hosts.⁴ The identification of horse-acquired bloodmeals is not surprising considering the close proximity of the study site to a horse farm. However, the findings are significant because of the high risk associated with EEEV infection and underscore the importance of vaccinating horses against EEEV. There has never been a reported case of human EEE in Tennessee, but the human bloodmeal identified from *Cx. nigripalpus* in this study highlights the lingering threat EEEV poses to humans in the area.

Persistent EEEV equine outbreaks in conjunction with elusive virus isolates indicate an ecologic system unique from typical transmission settings where virus readily circulates. Without minimum infection rate (MIR) data, it can be inferred as to which mosquitoes were likely involved in transmission based on feeding patterns, abundance, competence, flight range, and history of virus isolations. Results from previous EEEV studies have been combined with data from this study to assess the relative potential of each mosquito species at the outbreak site to act as an enzootic or epizootic vector of EEEV (Table 2).

Culex erraticus and *An. quadrimaculatus* are both competent EEEV transmitters,^{16,17} have frequently been sources of virus isolations,^{9-11,18,19} and were abundant at the outbreak site. However, *An. quadrimaculatus* had minimal interaction with the avian population, acquiring only 7% of its bloodmeals from avian hosts, 63% of which were from the mourning dove, an incompetent host of EEEV,²⁰ making it an unlikely vector (Table 2). In contrast, *Cx. erraticus* acquired 16% of its bloodmeals from avians, 7% from turtles and snakes, which have been identified as possible overwintering hosts of EEEV in the eastern United States,^{11,21,22} and 7% from small mammals, which are amplifier hosts of Venezuelan equine encephalitis and have been shown to exhibit key attributes of effective amplifier hosts of EEEV, including strong viremia production after infection (Arrigo and others, unpublished data) and virus isolations in the field.^{23,24} Ectotherms, in particular, have recently elevated their status as potential reservoir hosts of EEEV in the southeastern United States because of their large contribution to vertebrate biomass in terrestrial ecosystems and targeted feeding by *Culex* mosquitoes.^{25,26} These data suggest *Cx. erraticus* has strong potential for involvement in EEEV transmission at the study site as both an enzootic and epizootic vector (Table 2).

Culex nigripalpus exists predominantly in the southern states below Tennessee, yet its distribution briefly extends north through the western region of the state, including the outbreak site in Chester County.²⁷ This species was not found in high numbers in the CDC light traps, yet it likely plays a more defining role in west Tennessee than at other Tennessee EEEV outbreak sites. Its preference for hot and humid

TABLE 1
Number and percentage of vertebrate hosts identified from blood fed mosquitoes*

| | <i>Cx. erraticus</i> | | <i>An. quadrimaculatus</i> | | <i>Cx. nigripalpus</i> | | <i>An. punctipennis</i> | | <i>Cs. melanura</i> | |
|-----------------------------|----------------------|---------------------------|----------------------------|----------------------------|------------------------|---------------------------|-------------------------|---------------------------|---------------------|--------------------------|
| | No. | Percent of total (N = 82) | No. | Percent of total (N = 112) | No. | Percent of total (N = 30) | No. | Percent of total (N = 34) | No. | Percent of total (N = 3) |
| Mammalian | | | | | | | | | | |
| White-tailed deer | 49 | 60 | 45 | 40 | 8 | 27 | 10 | 29 | – | – |
| Horse | 7 | 9 | 53‡ | 47 | 9 | 30 | 24 | 71 | – | – |
| Swamp rabbit | 4 | 5 | 2 | 2 | – | – | – | – | – | – |
| Eastern cottontail | 1 | 1 | – | – | – | – | – | – | – | – |
| Cow | 1 | 1 | 2 | 2 | – | – | – | – | – | – |
| Cotton rat | 1 | 1 | – | – | – | – | – | – | – | – |
| River otter | – | – | 1 | 1 | – | – | – | – | – | – |
| Armadillo | – | – | 1 | 1 | – | – | – | – | – | – |
| Human | – | – | – | – | 1 | 3 | – | – | – | – |
| Raccoon | – | – | – | – | 1 | 3 | – | – | – | – |
| Totals | 63 | 77% | 104 | 93% | 19 | 63% | 34 | 100% | 0 | 0% |
| Avian | | | | | | | | | | |
| House finch | 4 | 5 | 3 | 3 | – | – | – | – | – | – |
| Carolina wren | 4 | 5 | – | – | 1 | 3 | – | – | – | – |
| Tawny owl (91%)† | 2 | 2 | – | – | 2 | 7 | – | – | – | – |
| Yellow-billed cuckoo | 1 | 1 | – | – | 1 | 3 | – | – | – | – |
| Northern cardinal | 1 | 1 | – | – | – | – | – | – | 1 | 33 |
| Green heron | 1 | 1 | – | – | 1 | 3 | – | – | – | – |
| Mourning dove | – | – | 5‡ | 4 | – | – | – | – | – | – |
| Yellow-crowned night heron | – | – | – | – | 1 | 3 | – | – | – | – |
| Grey catbird | – | – | – | – | 1 | 3 | – | – | – | – |
| Indigo bunting | – | – | – | – | 1 | 3 | – | – | – | – |
| PR vireo (96%) ^a | – | – | – | – | – | – | – | – | 1 | 33 |
| Yellow-rumped warbler | – | – | – | – | – | – | – | – | 1 | 33 |
| Totals | 13 | 16% | 8 | 7% | 8 | 27% | 0 | 0% | 3 | 100% |
| Reptilian | | | | | | | | | | |
| Eastern box turtle | 5 | 6 | – | – | 3 | 10 | – | – | – | – |
| Eastern racer | 1 | 1 | – | – | – | – | – | – | – | – |
| Totals | 6 | 7% | 0 | 0% | 3 | 10% | 0 | 0% | 0 | 0% |

* Only mosquito species from which at least three blood meals were identified are represented in this table.

† Sequences did not generate a strong enough match in the BLAST database to provide an unambiguous identification beyond genus.

‡ Two blood meals obtained from a single mosquito.

conditions²⁸ combined with current climate change could lead to expansion of its range, providing opportunity for greater involvement in transmission throughout Tennessee. Its high proportion of avian feeding (27%) and occasional reptilian feeding (10%) make it an interesting species to examine further for its role in EEEV in Tennessee.

Evidence of avian feeding by a mosquito species is a strong indicator of involvement in EEEV transmission. Avian blood-meals were not identified from *An. crucians* in this study, but occasional avian feeding has been documented.⁵ *Cx. erraticus* and *Cx. nigripalpus* combined to feed on nine avian species,

supporting previous findings that they are catholic feeders.^{29–32} Furthermore, these two species have fed on birds thought to be competent for EEEV based on detection of either EEE virus or antibody, including the Carolina wren, northern cardinal, grey catbird, and green heron.^{33–35} The house finch accounted for 22% of all avian-acquired bloodmeals, and although it has not been evaluated as a host of EEEV, it has been implicated as a likely reservoir of Western equine encephalitis, a close relative of EEEV, in southern California.³⁶

This study was limited by poor resting box yields of certain species and low success rate of the PCR assay. A small

TABLE 2
Potential of mosquito species to serve as enzootic or epizootic vectors of EEE virus in Tennessee according to specific vector attributes

| Species | Competence* | Isolates† | Flight range‡ | Overall abundance§ | Host preference | Enzootic vector¶ | Bridge vector** |
|----------------------------|-------------|-----------|---------------|--------------------|-----------------|------------------|-----------------|
| <i>Cx. erraticus</i> | 2 | 2 | 1 | 3 | Opportunistic | 2 | 3 |
| <i>Cx. nigripalpus</i> †† | 1 | 1 | 2 | 1 | Opportunistic | 1 | 1 |
| <i>Cq. perturbans</i> | 1 | 3 | 2 | 2 | Opportunistic | 1 | 2 |
| <i>An. quadrimaculatus</i> | 2 | 1 | 1 | 2 | Mammals | 1 | 1 |
| <i>Cx. restuans</i> | 3 | 1 | 1 | 2 | Birds | 2 | 0 |
| <i>An. crucians</i> †† | 1 | 2 | 1 | 1 | Mammals | 0 | 1 |
| <i>Cs. melanura</i> | 3 | 3 | 3 | 0 | Birds | 1 | 0 |
| <i>Cx. pipiens</i> | 0 | 2 | 1 | 2 | Birds | 0 | 0 |
| <i>An. punctipennis</i> | 0 | 1 | 1 | 0 | Mammals | 0 | 0 |
| <i>Ae. vexans</i> | 0 | 2 | 3 | 1 | Mammals | 0 | 0 |

* Ability to be infected with and transmit EEEV in the laboratory: 0, none; 1, 1–10%; 2, 10–15%; 3, > 15%.^{16,17,42}

† Relative number of EEEV-positive pools detected: 0, never isolated; 1, rarely isolated; 2, occasionally isolated; 3, commonly isolated.^{6,9,11,18,19,43–46}

‡ Distance from forest margin when host seeking: 0, < 1 km; 1, 1–3 km; 2, 3–8 km; 3, > 8 km.^{45,47–51}

§ Population abundance in CDC light traps at epizootic site during summer of 2006: 0, < 1% of total; 1, 1–10%; 2, 10–20%; 3, > 20% (Mukherjee and others, unpublished data).

¶ Potential for species to be enzootic or maintenance vector: 0, no potential; 1, minor potential; 2, moderate potential; 3, major potential.

** Potential for species to serve as an epizootic vector: 0, no potential; 1, minor potential; 2, moderate potential; 3, major potential.

†† Competence experiments need to be performed for *Cx. nigripalpus* and *An. crucians*. We give them here a conservative score of 1 based on the large number of isolates from these species.

percentage of failed PCRs may be caused by insufficient blood volume in mosquito abdomens. For the remaining samples, there is little evidence that specific genera failed to amplify as a diversity of mammals, avians, and reptiles was identified. The low rate does suggest that bloodmeals potentially significant to the understanding of EEEV transmission could be missing from this analysis. For instance, we did not collect sufficiently blood fed *Cx. restuans* or *Cq. perturbans*. Further study into their roles in EEEV transmission is warranted because of these species' abundance in CDC light traps. Virus isolation from *Cq. perturbans* has been frequently documented, and it has been incriminated as an EEEV vector in Massachusetts, New Jersey, Ohio, and Alabama.^{6,11,37-39} Virus has also been isolated from *Cx. restuans*,¹¹ and it has been shown to transmit EEEV effectively in competence studies.¹⁷ It is an ornithophilic species and therefore likely to be involved in virus maintenance (Table 2).^{5,30,40} It will be useful to continue examination of feeding habits of these species to resolve any concern regarding the differential effects the undetected bloodmeals may have had on this study.

This is the first study to assess the feeding preferences of potential vectors at an EEE epizootic focus in Tennessee. It is interesting to note the unique nature of EEEV transmission dynamics in southeastern foci such as Tennessee where *Cs. melanura* may not play as essential a role as in other traditional transmission settings. *Cs. melanura* and *Cx. restuans* are likely enzootic vectors despite their low abundance and the absence of virus isolations. *Cx. erraticus* has emerged as a potentially important EEEV vector in the southeastern United States because of its abundance, history of virus isolations, and opportunistic feeding patterns⁹⁻¹¹ and may be essential for maintaining the enzootic cycle in Tennessee. It is the only mosquito species from which EEEV has been identified in Tennessee (Gottfried and others, unpublished data). It fed on a high proportion of avians, including species from which EEEV antibodies have been isolated, and along with *Cq. perturbans*, is a likely bridge vector to incidental hosts. *Cx. nigripalpus*, traditionally associated with EEE in Florida, is not strongly established in Tennessee,^{18,27,28,41} and the finding of high proportions of avian feeding in this species will make it interesting to study further as it potentially increases its distribution. *An. quadrimaculatus*, although highly abundant at the site, fed on a low proportion of avians and was an unlikely bridge vector during the 2005 epizootic.

Received July 14, 2008. Accepted for publication February 26, 2009.

Acknowledgments: The authors thank Claude Bailey at the Tennessee Department of Environment and Conservation for analysis of the study sites and Dr. Goudarz Molaei for providing control samples. We acknowledge Christina Moore and Sheri Roberts for assisting with sequencing reactions.

Financial support: This research was supported by a grant from the Southeast Center for Emerging Biological Threats (SECEBT). SC was supported by an appointment to the Emerging Infectious Diseases (EID) Fellowship Program administered by the Association of Public Health Laboratories (APHL) and funded by the Centers for Disease Control and Prevention (CDC).

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