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MOLECULAR IDENTIFICATION OF BLOOD-MEAL SOURCES IN *CULISETA MELANURA* AND *CULISETA MORSITANS* FROM AN ENDEMIC FOCUS OF EASTERN EQUINE ENCEPHALITIS VIRUS IN NEW YORK

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Abstract. Eastern equine encephalitis (EEE) virus perpetuates in an enzootic cycle involving ornithophilic mosquito vectors, principally *Culiseta melanura* (Coquillett) and avian amplification hosts. To better understand the role of *Cs. melanura* and *Culiseta morsitans* (Theobald) in the epizootiology of EEE virus, we collected blood-fed mosquitoes between 31 May and 15 October 2004 at two sites associated with an EEE virus focus in central New York and identified the source of vertebrate blood by nucleotide sequencing of polymerase chain reaction (PCR) products of the *cytochrome b* gene. Analysis of 484 *Cs. melanura* and 122 *Cs. morsitans* revealed that 94.2% and 86.9%, respectively, acquired blood solely from avian hosts. Blood meals derived exclusively from mammals were detected in 0.8% of *Cs. melanura* and 1.6% of *Cs. morsitans*. Individual mosquitoes containing mixed-blood meals from both avian and mammalian hosts were also detected in 5.0% of *Cs. melanura* and 11.5% of *Cs. morsitans*. Wood thrush constituted the most common vertebrate host for *Cs. melanura* (23.6%) and *Cs. morsitans* (30.9%), followed by American robin, song sparrow, ovenbird, red-eyed vireo, and common yellowthroat. Mammalian-derived blood meals were identified as white-tailed deer, horse, domestic cat, and eastern pipistrelle bat. There were three isolations of EEE virus from *Cs. melanura* and one from *Cs. morsitans.* These results suggest that wood thrush and a few other passerine birds may play key roles in supporting EEE virus transmission in the northeast and possibly throughout the geographic range of EEE in North America. The frequency of mammalian feedings also suggests that *Cs. melanura* and *Cs. morsitans* may play a role in the transmission of EEE virus to equines, in addition to maintaining enzootic transmission among avian hosts. We report the first isolation of arboviruses from mosquito vectors concomitant with the identifications of their blood meal sources.

INTRODUCTION

Eastern equine encephalitis (EEE) is a highly pathogenic arthropod-borne virus that perpetuates in an enzootic cycle involving wild birds and ornithophilic mosquito vectors. In the northeastern United States, the two principal enzootic vectors are *Culiseta melanura* (Coquillett) and, to a lesser degree, *Culiseta morsitans* (Theobald).[1–5] These mosquitoes are particularly efficient vectors because they feed almost exclusively on avian hosts.[4,6,7] However, occasional feeding on mammals has been documented,[8–11] suggesting they may potentially serve as epizootic vectors as well.

Serologic surveys of wild birds indicate that many species, particularly members of Passeriformes inhabiting fresh water swamp foci, are exposed to EEE virus.[12–16] It is not known, however, which avian species are critical for virus amplification. Most studies on the host feeding patterns of *Cs. melanura* and *Cs. morsitans* have not identified specific avian hosts because they were based largely on identification of mosquito blood meals using a panel of broadly reactive antisera. Recent technological advances using polymerase chain reaction (PCR)-based methods have permitted the identification of both avian- and mammalian-derived blood meals to the species level for the first time.[17,18] These methods allow us to directly estimate vector contact with different bird species, which is essential to evaluating their relative importance as potential amplification hosts of EEE virus.

The current research initiative was undertaken to identify the specific avian hosts of *Cs. melanura* and *Cs. morsitans* and to clarify the role of these hosts in the epizootiology and ecology of EEE virus. Accordingly, blood-fed mosquitoes were collected between 31 May and 15 October 2004 at two sites associated with the Toad Harbor-Big Bay Swamp (THS), a known EEE virus focus in central New York.[19,20] Vertebrate blood meals were identified by sequencing PCR products of the *cytochrome b* gene of mitochondrial DNA.

MATERIALS AND METHODS

Mosquito collections. Mosquitoes were collected between 31 May and 15 October 2004 at two study sites, the swamp perimeter and the Village of Central Square, associated with the THS complex, Town of West Monroe, Oswego County, NY (Figure 1). These sites have been continuously monitored since 1977 as part of a research and regional EEE surveillance program (J. J. Howard, unpublished data). During the epizootics in 1990 and 1991, EEE virus was isolated from *Cs. melanura* and *Cs. morsitans* collected at both sites,[20] although the virus has not been detected in either site since 1996 despite continuous surveillance (J. J. Howard, unpublished data). Both sites were used in previous local host preference studies of *Culiseta.*[4,7]

Ten resting shelters (RSs) were set at each site according to the established protocol.[21] The swamp perimeter site (43°16′01″ N, 76°05′24″ W) comprises a stand of mature Eastern hemlock (*Tsuga canadensis*) and beech (*Fagus grandifolia*) on a peninsula that extends east ~100 m into the swamp (Figure 1). The 10 shelters were positioned on the west-facing slope of the peninsula with the openings toward the swamp. The Village site (43°16′43″ N, 76°08′08″ W) is 5 km west of the swamp site in a wooded area ~100 m east of an apartment complex where a young child was exposed to EEE virus in

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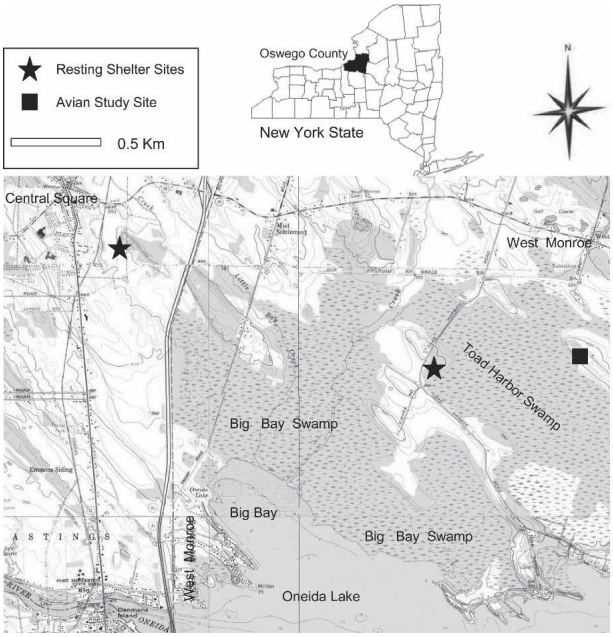

FIGURE 1. Location of resting shelter collection sites and avian study area at Toad Harbor–Big Bay Swamp complex, Oswego County, NY. The web site http://mapserver.maptech.com was used to create the figure.

1971.[22] Ten shelters were spaced between trees on the west edge of a plantation of white spruce (*Picea glauca*).

Mosquito collections and handling. Specimens were collected from the RS by chloroform anesthetization 2 consecutive days per week according to the following protocol. One to 2 mL of chloroform was placed on the box lid, the lid was fitted to the box, and the closed box laid upright (lid facing up). After closing four to five boxes, the units were opened, and the anesthetized mosquitoes were gently shaken onto a paper towel. The anesthetized blood-fed mosquitoes were transferred with forceps to a 120-mL glass bottle fitted with a plastic snap cap (Wheaton, Millville, NJ). The remaining mosquitoes were transferred into a separate 120-mL glass bottle. Each bottle was labeled by site and date. Generally, 90% or more of the specimens revived within 20 minutes of collecting from all 10 boxes. Bottles were placed in paper bags and stored in a cooler on wet ice. The collection bottles from the first day sampling of each week were held on wet ice for 24 hours until the next day's collection were made, after which specimens were transported to the NYS Department of Health Laboratory (NYSDOH) at the College of Veterinary Medicine, Cornell University, Ithaca, NY. Mosquitoes were cold-killed, identified to species, and examined for evidence of blood using a Nikon SMZ645 dissecting microscope using keys appropriate for the collection sites.[23] Non–blood-fed and gravid females and males were identified to species and enumerated. Data were recorded for date by species for the total collected from 10 shelters for the following three categories: non–blood-fed females, blood-fed and gravid females, and males. Yearly collections are expressed as the mean per shelter day (MSD; total collected/number of shelters collected per day). Only the blood-fed mosquitoes reported here were tested for viruses. Blood-fed mosquitoes were individually placed in 0.6-mL snap-capped microcentrifuge tubes and sealed with parafilm. Tubes were labeled with a numerical code representing the year and consecutive specimen number. Tubes containing *Culiseta* were stored in racks at –80°C until shipped on dry ice to the Connecticut Agricultural Experiment Station, New Haven, CT, for molecular analysis and virus isolation attempts.

Bird population. Bird population estimates were based on the analysis of mist-netting data from a 5-year (1986–1990) study on the avian hosts of EEE virus.[16] From May through September annually, birds were captured in mist nets set in the swamp, dry woods, an overgrown orchard, hedgerows, and field-edge ecotones, all within walking distance of the NYSDOH encephalitis field station[19] on the northern edge of THS (Figure 1). The field station (43°16′05″ N, 76°04′01″ W) is 2 km east of the perimeter resting box site. All birds were banded with US Fish and Wildlife Services bands. Data on species, age, sex, date, and location of capture were recorded by band number. Avian nomenclature followed the sixth edition of the American Ornithologists' Union[24] (AOU) and grouping of birds into orders and families followed the AOU classification.[24]

DNA isolation from blood-fed mosquitoes. With the aid of a dissecting microscope, mosquito abdomens were removed and reserved for blood-meal analysis. Each mosquito was dissected individually on a new microscope slide by using flame-sterilized forceps to avoid cross-contamination. DNA was isolated from the abdominal contents of blood-fed mosquitoes individually by using DNA-zol BD, (Molecular Research Center, Cincinnati, OH) according to the manufacturer's recommendation with some modifications.[18] Briefly, individual mosquito abdomens were homogenized with heat-sealed pipette tips in 1.5-mL tubes containing DNA-zol BD solution. The homogenates were incubated at room temperature for 5–10 minutes, mixed, and centrifuged at 10,000*g* for 10 minutes. DNA was precipitated by adding isopropanol and 3–4 μL Poly Acryl Carrier (Molecular Research Center). The DNA pellet was washed twice with 75% ethanol, air dried briefly, reconstituted in TE buffer (10 mmol/L Tris-HCl [pH 8.0], 1 mmol/L EDTA), and stored at –20°C for further analysis.

Blood-meal analysis. Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCR reactions. PCR primers were either based on a multiple alignment of cytochrome b sequences of avian and mammalian species obtained from GenBank or previously published primer sequences.[18] All DNA templates were initially screened with avian- and mammalian-specific primer pairs, and the sequences were analyzed. Avian-specific PCR primers were 5′-GAC TGT GAC AAA ATC CCN TTC CA-3′ (forward) and 5′-GGT CTT CAT CTY HGG YTT ACA AGA C-3′ (reverse) with amplified product size of 508 bp. PCR cycling condition included an initial reaction activation step at 95°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 50 seconds, and extension at 72°C for 40 seconds. The final cycle was completed with 7 minutes of extension at 72°C. Mammalian-specific PCR primers were 5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′ (forward) and 5′-TGT AGT TRT CWG GGT CHC CTA-3′ (reverse) with amplified product size of 772 bp. Initial PCR reaction activation step was performed at 95°C for 10 minutes followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 1.5 minutes. The final cycle

was completed with 7 minutes of extension at 72°C. In a few cases, other primer pairs were additionally used to resolve ambiguous sequences.[18] A *Taq* PCR Core Kit (Qiagen, Valencia, CA) was used for all PCR reactions according to the manufacturer's recommendation. A 50-μL reaction volume was prepared with 3 μL template DNA, 4 μL each primer (0.1–0.5 μmol/L), 5 μL 10× QIAGEN PCR Buffer (containing 15 mmol/L $MgCl_2$), 1 μL dNTP mix (10 mmol/L each), 0.25 μL *Taq* DNA Polymerase (1.25 U/reaction), and 32.75 μL water. PCR reactions were performed with the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using previously described thermal-cycling conditions.[18] PCR-amplified products were purified by using QIAquick PCR Purification Kit (Qiagen) and sequenced directly in cycle sequencing reactions by using the sequencer, 3730xl DNA Analyzer (Applied Biosystems) at the Keck Sequencing Facility, Yale University, New Haven, CT. Sequences were annotated by using ChromasPro version 1.22 (Technelysium Pty Ltd., Tewantin, Australia) and identified by comparison to the GenBank DNA sequence database (NCBI available online). The performance of the molecular-based assay was previously validated by isolating DNA from the blood of a number of known vertebrate species, subjecting them to PCR amplification and sequencing.[18]

Virus isolation and identification. The head and thorax of each blood-fed mosquito was processed for virus isolation by homogenizing them in 1 mL of phosphate-buffered saline containing 30% heat-inactivated rabbit serum, 0.5% gelatin, and 1× antibiotic/antimycotic by using a copper BB and vibration mill as previously described.[25] Mosquito homogenates were centrifuged at 4°C for 10 minutes at 520*g*, and 100 μL of the supernatant was inoculated onto a monolayer of confluent Vero cells growing in minimal essential media, 5% fetal bovine serum, and 1× antibiotic/antimycotic. Cells were maintained at 37°C in 5% CO_2 and examined daily for cytopathic effect from Day 3 through Day 7 after inoculation. RNA from infected cell supernatants was extracted by using a viral RNA kit (Qiagen) and screened for West Nile virus and EEE viruses by real-time RT-PCR assays.[26,27]

Statistical analysis. Mosquito and bird data were analyzed using SAS, Version 9.1 for windows (SAS Institute, Cary, NC). Within- and between-year captures of individual birds were tracked through the creation of two variables as described.[16] Briefly, the two variables were the number of captures this year (CTY) and number of total captures (CTOT). The first time a bird was captured and banded, the value of CTY and CTOT were each 1. At each recapture during the same year, the values of each were increased by one. Thus, a bird captured four times in 1 year had CTY and CTOT values of 4. If a bird was recaptured in a year subsequent to the banding year, the CTY value was reset to 1 but the CTOT was increased by 1. The number of individuals on site for the 5-year study was based on the number of birds that were first banded during the study (i.e., where CTY and CTOT = 1) plus the first between-year capture of any bird banded in a previous year (i.e., where CTY = 1 and CTOT > 1). χ^2 analysis for blood feeding pattern by site and species was performed online at http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html. Analysis of frequencies for blood-meal source and individual bird captures for passerines also used χ^2 with $\alpha = 0.05$ (SAS version 9.1 for Windows).

RESULTS

Mosquito collections. Between 31 May and 15 October 2004, mosquitoes were collected twice per week for a total of 26 days from the swamp perimeter and 25 days from the Village site. Collections contained *Cs. melanura, Cs. morsitans, Anopheles punctipennis* (Say), *Anopheles quadrimaculatus* Say, and *Culex territans* Walker. At the swamp perimeter, the MSDs for *Cs. melanura* were 6.01, 1.48, and 13.46 for non–blood-fed females, blood-fed and gravid females, and males, respectively. For *Cs. morsitans,* the MSDs were 1.98, 0.51, and 0.96, respectively. At the Village site, the means for *Cs. melanura* were 1.91, 0.87, and 0.87, and for *Cs. morsitans,* the means were 2.34, 0.65, and 1.69, respectively. In the previous 27 years, the number of days collections were made at these sites each season has ranged between 20 and 106 for the swamp perimeter and 18 and 56 days for the Village site. The MSDs for 2004 are consistent with means calculated for previous years (J.J.H., unpublished data). There were a total of 513 blood-fed *Cs. melanura* and 125 *Cs. morsitans* in the collections. Of these, there were 307 *Cs. melanura* collected from the swamp site and 206 from the Village site, and for *Cs. morsitans,* there were 35 and 90 blood-fed specimens collected, respectively, from the two sites.

DNA analysis. Blood-meal sources were successfully identified by DNA sequencing from 484 of 513 *Cs. melanura* and 122 of 125 *Cs. morsitans* (Table 1). Of the 484 *Cs. melanura* analyzed, 456 (94.2%) contained solely avian blood, 4 (0.8%) contained mammalian blood, and 24 (5%) had both avian and mammalian blood. Of the 122 *Cs. morsitans* analyzed, 106 (86.9%) contained avian blood, 2 (1.6%) contained mammalian blood, and 14 (11.5%) contained both avian and mammalian blood.

An analysis of 508 avian and mammalian blood-meal sources for *Cs. melanura* is shown in Table 2. We identified 52 species as avian hosts for *Cs. melanura*. A majority of the bloods ($N = 120$, 23.6%) were from wood thrush, *Hylocichla mustelina*, followed by American robin, *Turdus migratorius* ($N = 46$, 9.1%), song sparrow, *Melospiza melodia* ($N = 42$, 8.3%), ovenbird, *Seiurus aurocapilla* ($N = 30$, 5.9%), and red-

TABLE 1

Number and percentage of avian, mammalian, and mixed blood meals identified from *Cs. melanura* and *Cs. morsitans* collected at two sites associated with the Toad Harbor–Big Bay swamp in central New York

Species	Village			Swamp perimeter		
	Avian No. (%)	Mammalian No. (%)	Mixed* No. (%)	Avian No. (%)	Mammalian No. (%)	Mixed* No. (%)
Culiseta melanura	117 (92.8)	1 (0.5)	13 (6.8)	279 (95.2)	3 (1.0)	11 (3.8)
Culiseta morsitans	79 (88.7)	1 (1.1)	9 (10.1)	27 (81.8)	1 (3.0)	5 (15.2)

* All mixed meals contained DNA from both avian and mammalian hosts.

TABLE 2

Number and percentage of avian and mammalian blood meals identified from *Cs. melanura* collected in 2004 from two sites associated with Toad Harbor–Big Bay Swamp

	No.*	Percent of total
Avian hosts		
Wood thrush,† *Hylocichla mustelina*	120	23.6
American robin, *Turdus migratorius*	46	9.1
Song sparrow,†‡ *Melospiza melodia*	42	8.3
Ovenbird,† *Seiurus aurocapilla*	30	5.9
Red-eyed vireo,‡ *Vireo olivaceus*	29	5.7
Scarlet tanager,† *Piranga olivacea*	20	3.9
Common yellowthroat,†‡ *Geothlypis trichas*	18	3.5
Baltimore oriole, *Icterus galbula*	16	3.1
Black-capped chickadee,†‡ *Poecile atricapillus*	15	3.0
Veery,‡ *Catharus fuscescens*	14	2.8
Mourning dove,† *Zenaida macroura*	12	2.4
Cedar waxwing, *Bombycilla cedrorum*	10	2.0
Bobolink, *Dolichonyx oryzivorus*	10	2.0
Red-winged blackbird,† *Agelaius phoeniceus*	9	1.8
Rose-breasted grosbeak, *Pneucticus ludovicianus*	8	1.6
Gray catbird,†‡ *Dumetella carolinensis*	7	1.4
Northern cardinal,† *Cardinalis cardinalis*	5	1.0
Indigo bunting, *Passerina cyanea*	5	1.0
Yellow-throated vireo, *Vireo flavifrons*	5	1.0
Green heron, *Butorides virescens*	3	0.6
Black-billed cuckoo, *Coccyzus erythropthalmus*	3	0.6
Eastern towhee, *Pipilo erythrophthalmus*	3	0.6
American woodcock, *Scolopax minor*	3	0.6
Northern waterthrush, *Seiurus noveboracensis*	3	0.6
American redstart, *Setophaga ruticilla*	3	0.6
Wood duck, *Aix sponsa*	2	0.4
American bittern, *Botaurus lentiginosus*	2	0.4
American crow, *Corvus brachyrhynchos*	2	0.4
Yellow-rumped warbler, *Dendroica coronata*	2	0.4
Chestnut-sided warbler, *Dendroica pensylvanica*	2	0.4
Yellow warbler,† *Dendroica petechia*	2	0.4
Least flycatcher,‡ *Empidonax minimus*	2	0.4
Black-and-white warbler, *Mniotilta varia*	2	0.4
Brown-headed cowbird,† *Molothrus ater*	2	0.4
House sparrow, *Passer domesticus*	2	0.4
Savannah sparrow, *Passerculus sandwichensis*	2	0.4
Tree swallow,† *Tachycineta bicolor*	2	0.4
House wren,‡ *Troglodytes aedon*	2	0.4
Warbling vireo,† *Vireo gilvus*	2	0.4
Blue-winged teal, *Anas discors*	1	0.2
Mallard, *Anas platyrhynchos*	1	0.2
Tufted titmouse, *Baeolophus bicolor*	1	0.2
Great horned owl, *Bubo virginianus*	1	0.2
Hermit thrush, *Catharus guttatus*	1	0.2
Chimney swift, *Chaetura pelagica*	1	0.2
Eastern wood-Pewee, *Contopus virens*	1	0.2
Blue jay, *Cyanocitta cristata*	1	0.2
Black-throated green warbler,† *Dendroica virens*	1	0.2
White-winged crossbill, *Loxia leucoptera*	1	0.2
Common grackle, *Quiscalus quiscula*	1	0.2
Eastern bluebird, *Sialia sialis*	1	0.2
European starling, *Sturnus vulgaris*	1	0.2
Mammalian hosts		
White-tailed deer,† *Odocoileus virginianus*	18	3.5
Horse,† *Equus caballus*	9	1.8
Cat, *Felis catus*	1	0.2

* Includes 24 specimens from which double blood meals were identified.
† Species found with mixed blood meals.
‡ Species from which EEE virus has been isolated at Toad Harbor Swamp.

eyed vireo, *Vireo olivaceus* ($N = 29$, 5.7%). The 52 species were members of eight avian orders, but the majority were species from the order Passeriformes, 80.8% ($N = 42$), followed by Anseriformes (5.8%; $N = 3$), Ciconiiformes (3.8%; $N = 2$), and 1.9% ($N = 1$) each of the Apodiformes, Charadriiformes, Columbiformes, Cuculiformes, and Strigiformes. Mammalian hosts of *Cs. melanura* were identified as white-tailed deer, *Odocoileus virginianus* ($N = 18$, 3.5%), domestic horse, *Equus caballus* ($N = 9$, 1.8%), and domestic cat, *Felis catus* ($N = 1$, 0.2%). There were no significant differences between the two collection sites in overall composition of blood meals acquired by *Cs. melanura* from avian, mammalian, or mixed sources (χ^2, 2 df, $P \leq 1.0$).

The composition of 136 avian- and mammalian-derived blood meals for *Cs. morsitans* is shown in Table 3. We identified 21 species of avian hosts for *Cs. morsitans*. A majority of the bloods were from wood thrushes, ($N = 42$, 30.9%), followed by common yellowthroats, *Geothlypis trichas* ($N = 10$, 7.4%), red-eyed vireos, ($N = 9$, 6.6%), and 8 each (5.9%) from song sparrows and American robins. Mammalian hosts for *Cs. morsitans* were white-tailed deer ($N = 10$, 7.4%), horse ($N = 5$, 3.7%), and an eastern pipistrelle bat, *Pipistrellus subflavus* (0.7%). There were no significant differences between the two collection sites in overall composition of blood meals acquired by *Cs. morsitans* from avian, mammalian, or mixed sources (χ^2, 2 df, $P \leq 1.0$).

Avian populations. *Culiseta* fed on 56 species representing eight avian orders, but 60% of blood meals were derived from only six avian hosts: wood thrush ($N = 162$, 25.2%), American robin ($N = 54$, 8.4%), song sparrow ($N = 50$, 7.8%), red-eyed vireo ($N = 38$, 5.9%), ovenbird ($N = 34$, 5.3%), and common yellowthroat ($N = 28$, 4.3%).

From 1986 to 1990, there were a total of 6,906 captures of 5,296 individuals of 96 species. A majority of the species ($N = 81$, 84.4%) and captures ($N = 5,121$, 96.7%) were members of

TABLE 3

Number and percentage of avian and mammalian blood meals identified from *Cs. morsitans* collected in 2004 from two sites associated with Toad Harbor Swamp

	No.*	Percent of total
Avian hosts		
Wood thrush,† *Hylocichla mustelina*	42	30.9
Common yellowthroat,†‡ *Geothlypis trichas*	10	7.4
Red-eyed vireo,‡ *Vireo olivaceus*	9	6.6
Song sparrow,†‡ *Melospize melodia*	8	5.9
American robin,† *Turdus migratorius*	8	5.9
Gray catbird,†‡ *Dumetella carolinensis*	7	5.1
Mourning dove,† *Zenaida macroura*	6	4.4
Black-capped chickadee,‡ *Poecile atricapillus*	4	2.9
Ovenbird, *Seiurus aurocapilla*	4	2.9
Veery,‡ *Catharus fuscescens*	3	2.2
Scarlet tanager,† *Piranga olivacea*	3	2.2
Cooper's hawk, *Accipiter cooperii*	2	1.5
Bobolink, *Dolichonyx oryzivorus*	2	1.5
Baltimore oriole, *Icterus galbula*	2	1.5
Wild turkey, *Meleagris gallopavo*	2	1.5
American woodcock, *Scolopax minor*	2	1.5
European starling, *Sturnus vulgaris*	2	1.5
Cedar waxwing, *Bombycilla cedrorum*	1	0.7
Black-billed cuckoo, *Coccyzus erythropthalmus*	1	0.7
Blue-winged warbler, *Vermivora pinus*	1	0.7
Yellow-throated vireo,† *Vireo flavifrons*	1	0.7
Mammalian hosts		
White-tailed deer,† *Odocoileus virginianus*	10	7.4
Horse,† *Equus caballus*	5	3.7
Eastern pipistrelle bat, *Pipistrellus subflavus*	1	0.7

* Includes 14 specimens from which double blood meals were identified.
† Species found with mixed blood meals.
‡ Species from which EEE virus has been isolated at Toad Harbor Swamp.

the Passeriformes. The remaining captures were from the Piciformes (N = 123, 2.3%), Apodiformes (N = 21, 0.4%), Cuculiformes (N = 13, 0.2%), Charadriiformes (N = 10, 0.2%), 4 (<0.1%) each of the Falconiformes and Galliformes, and 1 Columbiformes. The 83 species of Passeriformes were members of 20 families, but > 75% of all captures were species from five Passerine families: Parulidae (warblers; N = 1,138, 22.2%), Emberizidae (sparrows; N = 949, 18.5%), Turidae (thrushes; N = 771, 15.1%), Mimidae (mockingbirds; N = 756, 14.8%), and Tyrannidae (flycatchers; N = 324, 6.3%). There were 4,956 birds captured only once and 340 between-year recaptures. Of the 5,296 individuals captured, 4,956 were only captured once (CTY and CTOT = 1), and 340 were captured in a year after their initial capture (CTY = 1 and CTOT > 1). The remaining captures were within-year recaptures of previous banded birds. Of these 5,296 individuals, 4,846 were (91.2%) were passerines in 18 families.

We compared the proportion of host blood meals by family for the 558 *Cs. melanura* and *Cs. morsitans* that had fed on passerines with the proportion of individuals during 1986 to 1990 (Table 4). The proportion of mosquitoes that had fed on wood thrushes and other members of the family Turidae (42.1%; American robin, veery, hermit thrush, and Eastern bluebird) was significantly higher than would be expected based on their proportion of the netted population (cell χ^2 = 165.52, deviation = 131.12; Table 4). This was also true for the number of host blood meals from the Icteridae (blackbirds), Thrupidae (tanagers), and Vireonidae (vireos), although the proportions for these families were not as great for the Turidae (Table 4). Families where the number of blood meals was lower than expected based on species abundance were the Mimidae (mockingbirds), Emberizidae (sparrows), Parulidae (warblers), Paridae (chickadees), and Bombycillidae (waxwings; Table 4). The most under-represented family was the Mimidae (cell χ^2 = 53.97, deviation = –65.51; Table 4), although the gray catbird was the most frequently netted bird in both multi-year bird studies conducted on site.[13,16] The second highest netted species during these studies was the song sparrow, but the Emberizidae was also significantly under-represented as a host blood-meal source (cell χ^2 = 22.85, deviation = –48.67; Table 4).

Virus isolations. There were four isolations of EEE virus from 513 *Cs. melanura* and 125 *Cs. morsitans* blood-fed mosquitoes. Two were isolated from *Cs. melanura* and one from a *Cs. morsitans* collected at the Village site, and one was from a *Cs. melanura* collected at the swamp perimeter site. Infected *Cs. melanura* were collected on 25 August (one at each site) and 8 September (from the Village site). The infected *Cs. morsitans* was collected at the Village site on 14 September. Sources of blood meals from EEE virus–infected *Culiseta* were identified as wood thrush, song sparrow, and ovenbird. West Nile virus was also isolated from a *Cs. melanura* collected at the Village site on 10 August, and it had fed on a red-eyed vireo.

DISCUSSION

Our study provides insight into the host associations of *Cs. melanura* and *Cs. morsitans* in this region of the northeastern United States. We found that these mosquitoes feed primarily on passerine birds and focus their feeding activity on several species including wood thrush, American robin, song sparrow, ovenbird, and red-eyed vireo that could support EEE virus transmission. A moderate proportion of *Cs. melanura* (5.0%) and *Cs. morsitans* (11.5%) acquired mixed blood meals from both avian and mammalian sources, suggesting that they could also facilitate transmission of EEE virus to incidental hosts such as horses and possibly humans. For the first time, we report the isolation of arboviruses from vector mosquitoes concomitant with the identification of the wild vertebrate hosts on which they had fed.

Our findings that > 90% of blood-fed *Cs. melanura* acquired blood meals from avian hosts and that 80% of blood meals were derived from passerines are consistent with the results of other studies that have used precipitin testing,[6–10] polyclonal antibodies,[28] or PCR and PCR–heteroduplex reactions.[17,29,30] Our PCR-based method took advantage of the conservation and diversity of the *cytochrome b* gene of the mitochondrial sequences as a useful marker in identifying the source of vertebrate blood from mosquitoes to the species level. While this method offers clear advantages over serologic identification of blood meals, a small percentage of the avian samples did not exactly match any of the DNA sequences currently available in the GenBank database. Evidence also suggests that a single mosquito could have ac-

TABLE 4

Comparison by passerine families of host blood meals for 558 blood-fed *Culiseta* and 4,846 individual birds netted at Toad Harbor Swamp 1986–1990

Passerine family		Host blood source				Individuals captured		
Scientific name	Common name	No.	Percent of total	Cell χ^2	Deviation	No.	Percent of total	Cell χ^2
Turdidae	Thrushes	235	42.11	165.52	131.12	771	15.91	19.06
Parulidae	Warblers	78	13.98	3.78	–19.16	863	17.81	0.45
Emberizidae	Sparrows	55	9.86	22.85	–48.67	949	19.58	2.63
Vireonidae	Vireos	46	8.24	9.33	16.57	239	4.93	1.07
Icteridae	Blackbirds	42	7.53	38.18	25.27	120	2.48	4.40
Thrupidae	Tanagers	23	4.12	80.32	18.66	19	0.39	9.25
Paridae	Chickadees	20	3.58	2.33	–8.08	252	5.20	0.27
Cardinialidae	Cardinals	18	3.23	26.73	12.32	37	0.76	3.08
Mimidae	Mockingbirds	14	2.51	53.97	–65.51	756	15.60	6.21
Miscellaneous families†		16	2.87	37.57	–49.67	620	12.79	4.33
Bombycillidae	Waxwings	11	1.97	6.92	–12.85	220	4.54	0.80

* Total χ^2 499.0317, 10 df, α = 0.05, $P < 0.001$.

† Combined species for Passerine families Certhiidae (creepers), Hirundinidae (swallows), Passeridae (house sparrow), Regulidae (kinglets), Sittidae (nuthatches), Sturnidae (starlings), Trogllodytidae (wrens), and Tyrannidae (flycatchers).

quired blood meals from two avian or two mammalian species; however, further modifications of the technique may be required to determine the source of blood meals to the species level in the aforementioned categories. The amount of blood acquired by mosquitoes, the time between capturing mosquitoes and processing for blood meal analysis, quality of isolated DNA, availability of the species-specific *cytochrome b* gene sequences in the database, the degrees of sequence homology among the vertebrate hosts, particularly bird species present in the study area, and the possibility of mixed blood meals either from two avian or mammalian species are among the factors that contribute to successful identification of the blood-meal source.

Our results also confirm the ornithophilic blood feeding pattern of *Cs. morsitans*.[4,10] We found that *Cs. melanura* or *Cs. morsitans* fed on 56 avian species representing eight orders, and whereas Passeriformes comprised > 80% of all avian blood meals, the wood thrush represented 24% of the total blood meals and 27% of the blood meals from passerines. The predominance of wood thrushes as a host suggests a specific host preference rather than opportunistic feeding based on avian species abundance.[9,29] Virtually all avian host species identified as blood-meal sources have been reported from THS based on mist netting records[13,16] or direct observation.[19] However, the proportion of mosquitoes that fed on wood thrushes and other members of the family Turidae (American robin, veery, hermit thrush, and Eastern bluebird) is significantly higher than would be expected based on their proportion of the netted population (Table 4). The underrepresentation of the Mimidae and Emberizidae was unexpected because gray catbirds and song sparrows are the two most frequently netted species at THS.[16] Although mist-netting results are biased estimates of the avian population because they underestimate canopy species,[31] they provide reasonable estimates of avian abundance at 2- to 3-m heights. Both *Cs. melanura* and *Cs. morsitans* prefer to host-seek at ground level in an open field.[32] Our avian population data were from a study completed 15 years ago, but the ecology of the THS complex has remained relatively unchanged since our original description,[19] because over two thirds of the area is a NYS game wildlife area. Furthermore, two long-term studies on the role of birds in the epizootiology of EEE virus have been conducted at THS.[13,16] In the earlier study, over 3 years (1978–1980), there were 4,272 individuals of 93 species banded and a total of 6,292 captures.[13] The species abundance for the two studies was similar, and some of the individuals banded during the first study were recaptured during the latter.[16] An interesting observation between the two studies is that no scarlet tanagers (Thrupidae) were banded during 1978–1980, whereas 19 individuals were banded between 1986 and 1990, and we herein report that 3.8% of *Culiseta* blood meals ($N = 604$) were from this species.

Many species of birds support enzootic transmission of EEE virus in North America, although the relative contribution of each species is unclear. We recently reported the current total is 66 species from which EEE virus has been isolated.[16] Studies have reported that certain species seem to be more important in the enzootic cycling of EEE virus and wood thrushes and other dominant species reported here are among that group. In Alabama, there were 42 isolations of EEE virus from > 3,000 birds bled, but there were more from wood thrush than any other species,[12] and one half the isolations were from four species: wood thrush ($N = 7$), gray catbird and veery ($N = 5$ each), and red-eyed vireo ($N = 4$). In New Jersey, early season virus isolations from a gray catbird (among others) and high EEE virus prevalence rates for summer residents, including the wood thrush (59.9%) American robin (30.4%), ovenbird (37.1%), red-eyed vireo (21.0%), and scarlet tanager (11.1%), were reported as evidence of a cryptic EEE virus cycle.[15] In Massachusetts, isolation of EEE virus from gray catbird and American robin has been reported,[14] but the highest EEE antibody prevalence rates were found in wood thrush (26.7%) followed by swamp sparrow (24.5%), American robin (20.9%), and ovenbird (18.2%).

Overall, the blood-feeding patterns reported here for *Cs. melanura* and *Cs. morsitans* are consistent with the results of other studies in indicating the importance of these species as enzootic hosts of EEE virus. Although *Cs. melanura* is largely ornithophilic, infrequent feeding on mammals is indicated by nearly 6% of *Culiseta* tested in this study. The only study reporting exclusive mammalian feeding by *Cs. melanura* was based on precipitin testing of specimens collected from the Pocomoke Swamp of Maryland.[33] The validity of this study, however, was later challenged because only 37% ($N = 130$) of the blood meals were identified, and none of the reactors were of avian origin.[34] A study conducted a few years later at the same swamp reported that 12.7% ($N = 1,556$) of *Cs. melanura* blood meals were of mammalian origin.[8] Recently, feeding on deer ($N = 3$), humans ($N = 2$), and a raccoon was reported for six *Cs. melanura* identified with non-avian hosts collected in New Jersey,[17] but most reports in the northeast indicate minimal mammalian blood feeding by *Cs. melanura* or *Cs. morsitans*.[4,7,10] Only three mammalian blood meals were identified from > 2,000 blood-fed *Cs. melanura* collected at sites in Oswego County including THS,[7] and there were only 12 blood meals identified as mammalian from > 3,000 *Cs. melanura* collected from sites associated with two swamps in southeastern Massachusetts.[10] Ours is the first report of *Cs. morsitans* feeding on a bat, although it was reported that both species were attracted to but did not feed on little brown bats (*Myotis lucifugus*) confined in lard can traps.[35]

Dual feeding on birds by *Cs. melanura* was shown in field experiments that used caged chickens. Eight percent ($N = 189$) of blood-fed *Cs. melanura* were found to have fed on blood containing both rubidium- and cesium-labeled blood.[36] Mixed blood meals from mammals and birds are more frequently reported than sole mammal feedings. In the Massachusetts study, the percent of mixed meals ranged from 0.04% ($N = 1,116$) to 11.0% ($N = 471$) depending on year and site. The highest percentage was from blood-fed *Cs. melanura* collected from the swamp perimeter where 21 bloods were a mix of passerine and rabbit and 32 were a mix of passerine and unspecified mammal.[10] However, our finding of mixed blood meals from avian and large mammalian hosts, horses and white-tailed deer, is unique and suggests that *Cs. melanura* or *Cs. morsitans* in the northeast may play a role in the transmission of EEE virus to horses. Results from a mark–recapture study[37] defined the area at risk to EEE based on the dispersal of *Cs. melanura* and *Cs. morsitans* and equine cases have occurred within definable limits of *Culiseta* breeding swamps in central New York.[20] It is more logical to infer that the reappearance of disease at the same site can be attributed to the dispersion of an infective vector species rather than the complex concept of primary and secondary or bridge

vectors interacting with avian hosts establishing cycling of EEE virus that leads to equine disease.[1–3] Our finding that *Culiseta* fed on white-tailed deer is also consistent with reports of EEE in free-living white-tailed deer in Houston County, GA,[38] and Kent and Montcalm counties, MI (Michigan Department of Health, unpublished data). In both epizootics, equine EEE was also recorded from these counties.

Isolation of viruses from vector mosquitoes concurrent with host identification of mosquito blood meals has not been reported in the literature. Virus was isolated from the head and thorax of blood-fed *Culiseta*, indicating that they were infected, but it cannot be determined if the mosquito acquired the virus from the current host or the previous gonotrophic cycle. While laboratory studies have shown the dissemination of EEE virus in *Cs. melanura* within 17 hours of feeding and the presence of virus within 48–72 hours of feeding on an infectious host,[3] most of the infected mosquitoes were collected from the Village during August and September when populations are dominated by older, previously fed females.[4,7] However, it is noteworthy that the host blood meals for the infected mosquitoes were from bird species that may be important avian hosts of EEE virus in central New York and possibly throughout the range of EEE virus in North America.

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REFERENCES

1. Hayes RO, 1981. Eastern and western encephalitis. Beran GW, ed. *Viral Zoonoses, Section B.* Vol. 1. Boca Raton, FL: CRC Handbook Series in Zoonoses, 29–57.
2. Morris CD, 1988. Eastern equine encephalomyelitis. Monath TP, ed. *Arboviruses Epidemiology and Ecology*. Vol. 3. Boca Raton, FL: CRC Press, 1–20.
3. Scott TW, Weaver SC, 1989. Eastern equine encephalomyelitis virus: Epidemiology and evolution of mosquito transmission. *Adv Virus Res 37:* 277–328.
4. Morris CD, Zimmerman RH, 1981. Epizootiology of eastern equine encephalomyelitis virus in upstate New York, USA. III. Population dynamics and vector potential of adult *Culiseta morsitans* (Diptera: Culicidae). *J Med Entomol 18:* 313–316.
5. Howard JJ, Grayson MA, White DJ, Morris CD, 1994. Eastern equine encephalitis in New York State. *J FL Mosq Cont Assoc 65:* 1–7.
6. Magnarelli LA, 1977. Host feeding patterns of Connecticut mosquitoes (Diptera: Culicidae). *Am J Trop Med Hyg 26:* 547–552.
7. Morris CD, Zimmerman RH, Edman JD, 1980b. Epizootiology of eastern equine encephalomyelitis virus in upstate New York, USA. II. Population dynamics and vector potential of adult *Culiseta melanura* (Diptera: Culicidae) in relation to distance from breeding site. *J Med Entomol 17:* 453–465.
8. Joseph SR, Bickley WE, 1969. *Culiseta melanura (Coquillett) on the Eastern Shore of Maryland (Diptera: Culicidae)*. University of Maryland Agricultural Experimental Station. Bull.A161, College Park, MD.
9. Edman JD, Webber LA, Kale HW II, 1972. Host-feeding patterns of Florida mosquitoes. II. *Culiseta. J Med Entomol 9:* 429–434.
10. Nasci RS, Edman JD, 1981. Blood-feeding patterns of *Culiseta melanura* (Diptera: Culicidae) and associated sylvan mosquitoes in southeastern Massachusetts eastern equine encephalitis enzootic foci. *J Med Entomol 18:* 493–500.
11. Hayes RO, Doance OW, 1958. Primary record of *Culiseta melanura* biting man in nature. *Mosq News 18:* 216–217.
12. Stamm D, 1968. Arbovirus studies in birds in South Alabama 1959–1960. *Am J Epidemiol 67:* 127–137.
13. Emord DE, Morris CD, 1984. Epizootiology of eastern equine encephalomyelitis in upstate, New York, USA. VI. Antibody prevalence in wild birds during an interepizootic period. *J Med Entomol 21:* 395–404.
14. Main AJ, Anderson KS, Maxfield HK, Rosenau B, Oliver C, 1988. Duration of *Alphavirus* neutralizing antibody in naturally infected birds. *Am J Trop Med Hyg 38:* 208–217.
15. Crans WJ, Caccamise DF, McNelly JR, 1994. Eastern equine encephalomyelitis virus in relation to the avian community of a coastal cedar swamp. *J Med Entomol 31:* 711–728.
16. Howard JJ, Oliver J, Grayson MA, 2004. Antibody response of wild birds to natural infection with *Alphaviruses. J Med Entomol 41:* 1090–1103.
17. Apperson CS, Hassan HK, Harrison BA, Savage HM, Aspen SE, Farajollahi A, Crans W, Daniels TJ, Falco RC, Benedict M, Anderson M, McMillen L, Unnasch TR, 2004. Host feeding patterns of established and potential mosquito vectors of West Nile virus in the eastern United States. *Vector Borne Zoonotic Dis 4:* 71–82.
18. Molaei G, Andreadis TG, Armstrong PM, Anderson JF, Vossbrinck CR, 2006. Host feeding patterns of *Culex* mosquitoes and West Nile virus transmission, northeastern United States. *Emerg Infect Dis 12:* 468–474.
19. Morris CD, Corey ME, Emord DE, Howard JJ, 1980a. Epizootiology of eastern equine encephalomyelitis virus in upstate New York, USA. I. Introduction, demography and natural environment of an endemic focus. *J Med Entomol 17:* 442–452.
20. Howard JJ, Grayson MA, White DJ, Oliver J, 1996. Evidence of multiple foci of eastern equine encephalitis virus (Togaviridae: *Alphavirus*) in central New York State. *J Med Entomol 33:* 421–432.
21. Morris CD, 1981. A structural and operational analysis of diurnal resting shelters for mosquitoes (Diptera: Culicidae). *J Med Entomol 18:* 419–424.
22. Morris CD, Whitney E, Bast TF, Deibel R, 1973. An outbreak of eastern equine encephalomyelitis in upstate New York during 1971. *Am J Trop Med Hyg 22:* 561–566.
23. Means RG, 1987. *Mosquitoes of New York. II. Genera of Culicidae other than Aedes occurring in New York.* Albany, NY: University of the State of New York.
24. American Ornithologists' Union, 1983. *Check-List of North American Birds.* Sixth edition. Lawrence, KS: American Ornithologist Union.
25. Andreadis TG, Anderson JF, Vossbrinck CR, Main AJ, 2004. Epidemiology of West Nile virus in Connecticut: A five-year analysis of mosquito data 1999–2003. *Vector Borne Zoonotic Dis 44:* 360–378.
26. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Sav-

age HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT, 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan Reverse Transcriptase-PCR Assay. *J Clin Microbiol 8:* 4066–4071.

27. Lambert AJ, Martin DA, Lanciotti RS, 2003. Detection of North American eastern and western equine encephalitis viruses by nucleic acid amplification assays. *J Clin Microbiol 41:* 379–385.
28. Irby WS, Apperson CS, 1988. Hosts of mosquitoes in the coastal plain of North Carolina. *J Med Entomol 25:* 85–93.
29. Hassan HK, Cupp EW, Hill GE, Katholi CR, Klingler K, Unnasch TR, 2003. Avian host preference by vectors of eastern equine encephalomyelitis virus. *Am J Trop Med Hyg 69:* 641–647.
30. Ngo KA, Kramer LD, 2003. Identification of mosquito blood meals using polymerase chain reaction (PCR) with order-specific primers. *J Med Entomol 40:* 215–222.
31. Bibby CJ, Burgess ND, Hill DA, Mustoe S, 2000. *Bird Census Techniques*. Second edition. New York: Academic Press.
32. Howard JJ, Emord DE, Morris CD, 1983. Epizootiology of eastern equine encephalomyelitis virus in upstate New York, USA. V. Habitat preference of host-seeking mosquitoes (Diptera: Culicidae). *J Med Entomol 20:* 62–69.
33. Moussa MA, Gould DJ, Nolan MP, Hayes DE, 1966. Observations on *Culiseta melanura* (Coquillett) in relation to encephalitis in southern Maryland. *Mosq News 26:* 385–393.
34. Hayes CG, Wallis RC, 1977. Ecology of western equine encephalomyelitis in the eastern United States. *Adv Virus Res 21:* 37–83.
35. Hayes RO, 1961. Host preferences of *Culiseta melanura* and allied mosquitoes. *Mosq News 22:* 352–356.
36. Anderson RA, Edman JD, Scott TW, 1990. Rubidium and cesium as host blood-markers to study multiple feeding by mosquitoes (Diptera: Culicidae). *J Med Entomol 27:* 999–1001.
37. Howard JJ, White DJ, Muller SL, 1989. Mark-recapture studies on the *Culiseta* (Diptera: Culicidae) vectors of eastern equine encephalitis virus. *J Med Entomol 26:* 190–199.
38. Tate CM, Howarth EW, Stalknecht DE, Allison AB, Fischer JR, Mead DG, 2005. Eastern equine encephalitis in a free-living white-tailed deer (*Odocoileus virginianus*). *J Wildlife Dis 41:* 241–245.