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ORIGINAL ARTICLE

Vector-Host Interactions and Epizootiology of Eastern Equine Encephalitis Virus in Massachusetts

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Abstract

Eastern equine encephalitis (EEE) virus is a highly pathogenic mosquito-borne zoonosis that is responsible for outbreaks of severe disease in humans and equines, resulting in high mortality or severe neurological impairment in most survivors. In the northeastern United States, EEE virus is maintained in an enzootic cycle involving the ornithophilic mosquito, *Culiseta melanura* (Coquillett) and passerine birds in freshwater swamp habitats. To evaluate the role of *Cs. melanura* and *Culiseta morsitans* (Theobald) in recent episodes of EEE virus activity in Massachusetts, we collected blood-fed mosquitoes between June, 2007, and October, 2008, from virus foci in 6 counties, and identified the source of blood meals by PCR amplification of mitochondrial cytochrome *b* gene and sequencing. Analysis of 529 *Cs. melanura* and 25 *Cs. morsitans* revealed that nearly 99% and 96% of mosquitoes, respectively, acquired blood meals solely from avian hosts. American Robin, *Turdus migratorius* Linnaeus was identified as the most common vertebrate host for *Cs. melanura* (21.7%, $n=115$), followed by Tufted Titmouse, *Baeolophus bicolor* (L.) (8.7%, $n=46$), Black-capped Chickadee, *Poecile atricapillus* (L.) (8.5%, $n=45$), Scarlet Tanager, *Piranga olivacea* (Gmelin) (6.8%, $n=36$), Field Sparrow, *Spizella pusilla* (Wilson) (6.2%, $n=33$), Northern Cardinal, *Cardinalis cardinalis* (L.) (5.7%, $n=30$), and other mostly Passeriformes birds. Mammalian-derived blood meals were identified as white-tailed deer, *Odocoileus virginianus* Zimmermann, domestic cow, *Bos taurus* L., and human, *Homo sapiens* L. There were 4 isolations of EEE virus, West Nile virus, and Highland J virus from *Cs. melanura*. Our results in conjunction with other lines of evidence, including reservoir competency, prevalence of antibody, and infection in nature, suggest that the American Robin, Tufted Titmouse, Black-capped Chickadee, and a few other passerine birds may play key roles in supporting EEE virus transmission in Massachusetts. Infrequent blood feeding of *Cs. melanura* on mammalian hosts, including humans, also indicates that this mosquito may occasionally contribute to epidemic/epizootic transmission of EEE virus in this region.

Key Words: *Culiseta melanura*—*Culiseta morsitans*—Blood feeding pattern—Mitochondrial cytochrome *b* gene—Epizootiology—Eastern equine encephalitis virus.

Introduction

Eastern Equine Encephalitis (EEE) virus (family Togaviridae, genus *Alphavirus*) is a highly pathogenic mosquito-borne agent responsible for periodic outbreaks of severe disease in humans and equines, causing high mortality and severe neurologic impairment in most survivors. During the last decade, episodes of EEE virus have reemerged in the northeastern United States, including Massachusetts, where there has been increased virus activity and recurrent human and equine cases (Centers for Disease Control and Prevention 2006). These episodes occur when ecological conditions favor virus amplification followed by overflow into human and equine populations.

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EEE virus is amplified in an enzootic cycle involving ornithophilic mosquitoes, principally *Culiseta melanura* (Coquillett), and to a lesser extent *Culiseta morsitans* (Theobald), and birds, primarily members of Passeriformes (perching birds) (Hayes et al. 1981, Morris and Zimmerman 1981, Morris 1988, Scott and Weaver 1989, Crans et al. 1994, Howard et al. 1994) inhabiting fresh water swamp foci. However, the role that these mosquitoes play in epidemic and epizootic transmission of virus to humans and horses, and the contribution of various bird species as amplification hosts is not well defined. The conventional paradigm posits that *Cs. melanura* and *Cs. morsitans* are involved in enzootic cycling of EEE virus among birds, whereas other mosquito species such as *Coquillettidia perturbans* (Walker), *Ochlerotatus canadensis* (Theobald), *Aedes vexans* (Meigen), and *Oc. sollicitans* (Walker) that feed more opportunistically, transmit virus to mammals, including humans and horses (Crans 1977, Crans and Schulze 1986).

However, recent host-feeding pattern studies indicate that populations of *Cs. melanura* in the northeastern United States acquire small proportions of blood meals from mammals in addition to birds as their preferred hosts (Molaei and Andreadis 2006, Molaei et al. 2006a). Furthermore, in a recent study that evaluated the ability of field-collected mosquitoes to acquire, replicate, and potentially transmit EEE virus, *Cs. melanura* had the highest prevalence of infection and virus titers among other potential vectors (Armstrong and Andreadis 2010). These research findings in concert with frequent isolations of EEE virus from field-collected *Cs. melanura* highlight the potential importance of this mosquito species as both primary enzootic and epidemic vector of the virus.

The current research initiative was undertaken to examine the vector–host interactions and blood-feeding patterns of *Cs. melanura* and *Cs. morsitans* and their role in enzootic and epidemic/epizootic transmission in a region with endemic EEE virus activity. Accordingly, engorged mosquitoes were collected during peak mosquito season from June to October of 2007 and 2008 from 6 Massachusetts counties with focal EEE virus activity. Blood meal sources were identified by sequencing PCR products of the mitochondrial cytochrome *b* gene.

Materials and Methods

Study sites

Mosquitoes were collected in 30 trapping sites located in Bristol, Essex, Middlesex, Norfolk, Plymouth, and Worcester Counties in Massachusetts (Fig. 1). Most of the area's estimated human population of 4,758,369 resides in regions consisting of cities and unincorporated communities. In general, the study area is highly urbanized, with a few remnants of agricultural and undisturbed natural landscapes interspersed within highly fragmented residential and commercial developments. Most collection sites were located along the borders of wooded wetland habitats dominated by red maple, *Acer rubrum* L., and Atlantic white cedar, *Chamaecyparis thyoides* (L.) Britton, Sterns & Poggenb trees. The majority of mosquitoes were collected within established and emergent EEE virus foci in Bristol, Plymouth, and Essex Counties.

Mosquito sampling

Engorged mosquitoes were collected weekly between June 4, 2007, and October 15, 2008, from locations within the 6-county study area (Fig. 1) by using primarily resting boxes according to established protocol (Morris 1981). Resting boxes were placed on dry forested uplands within sight of red maple swamp habitats surrounded by shrubs, on manicured garden areas under rhododendron bushes, in the middle of mixed forest in the proximity of pine swamps, and along the edges of Atlantic white cedar, red maple, and maple and high-bush blueberry swamps. Engorged mosquitoes were mostly sampled from locations where positive mosquito pools or human or equine cases of EEE virus had been previously reported. In addition to resting boxes, supplementary samplings also were made by using modified dry ice-baited, CDC-style light traps (John W. Hock Company, Gainesville, FL) (Sudia and Chamberlain 1962). These traps were set along the tree line of wooded Atlantic white cedar, maple, hemlock, and high-bush blueberry swamp areas. Specimens were transported alive in coolers (4–8°C) with ice packs to the various agency laboratories. Engorged mosquitoes were then speciated using a dissecting microscope and identification key (Andreadis et al. 2005). Specimens with visible blood meals were transferred to microtubes, labeled with a unique number, and transported on dry ice to the Massachusetts Department of Public Health to be held at −80°C in an ultra-low-temperature freezer. Blood meal analysis and detection of EEE virus from the engorged mosquitoes were performed at the Connecticut Agricultural Experiment Station (CAES).

DNA isolation and blood meal identification from engorged mosquitoes

Mosquito abdomens were removed with the aid of a dissecting microscope and disposable razor blades for blood meal analysis. DNA was isolated from the abdominal content of engorged mosquitoes individually by using DNAzol BD (Molecular Research Center, Cincinnati, OH) or DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturers' recommendation with some modifications as described elsewhere (Molaei et al. 2006a, 2006b). Isolated DNA from the mosquito blood meals served as DNA templates in subsequent PCR assays with primers based on vertebrate mitochondrial cytochrome *b* sequences using previously described protocols and thermal cycling conditions (Molaei et al. 2006a, 2006b). The source of mosquito blood meals was identified by sequence comparison to the GenBank DNA sequence database (NCBI at http://www.ncbi.nlm.nih.gov/).

Detection of virus in blood-fed mosquitoes

Blood-fed mosquitoes were tested for the presence of EEE virus or other arboviruses by virus isolation in cell culture (Armstrong et al. 2011) and with real-time RT-PCR assays (Lanciotti et al. 2000, Lambert et al. 2003). The head and thorax of individual blood-fed mosquitoes were homogenized in 1 mL of phosphate-buffered saline (PBS) containing 30% heat-inactivated rabbit serum, 0.5% gelatin, and antibiotic/antimycotic by using a Mixer Mill apparatus (model MM300, Retsch Inc., Haan, Germany), as previously described (Andreadis et al. 2004). Mosquito homogenates were centrifuged at 4°C for 10 min at $520 \times g$, and then 100 μL of the supernatant was inoculated into a 25-cm^2 flask containing Vero cells growing in minimal essential media, 5% fetal bovine serum, and antibiotics/antimycotics. Cells were maintained at 37°C

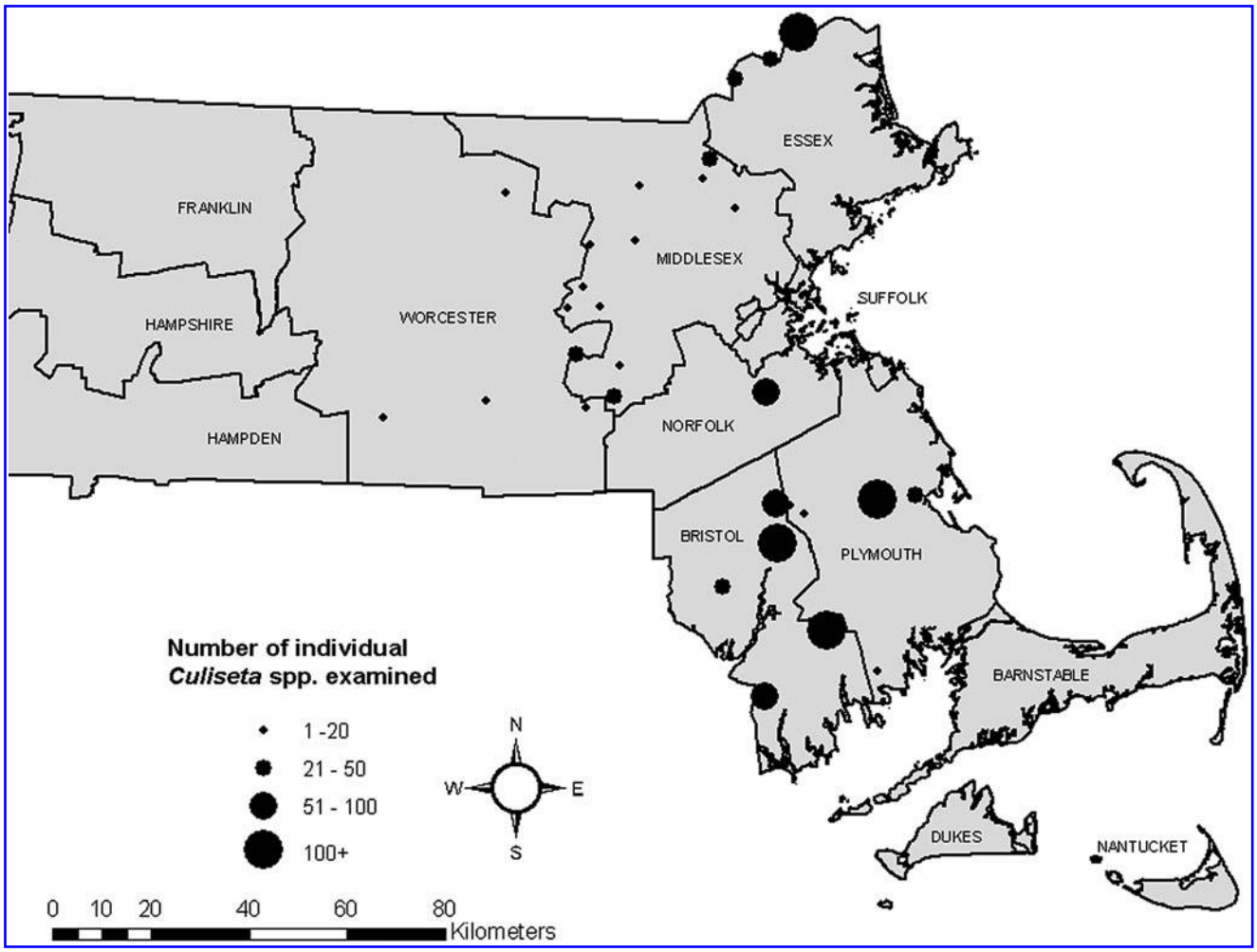

FIG. 1. Location of mosquito collection sites in Bristol, Essex, Middlesex, Norfolk, Plymouth, and Worcester Counties in Massachusetts.

in 5% CO_2 and examined daily for cytopathic effect (CPE) 3–7 days postinoculation. RNA was extracted from CPE-positive cell cultures by using the viral RNA Kit (Qiagen), and virus was identified by real-time RT-PCR.

Bird population estimates

Frequency estimates of avian species for Bristol, Essex, Middlesex, Norfolk, Plymouth, and Worcester Counties were performed by using data from the online eBird database (http://www.ebird.org/) developed in 2002 by the Cornell Laboratory of Ornithology and National Audubon Society to track bird distribution and abundance in North America. The observation frequency, expressed in decimal format ranging from 0 to 1, represents the percentage of checklists reporting the species within a specified date range and region (Table 1).

The estimated frequency data used consist of information obtained from 4918 checklists analyzed on a weekly basis during the mosquito collection season (June through November, 2007–2008). Bird species were ranked in descending order by frequency index, from most to least frequently observed, to assess relative distribution and observation frequency within the region, and for comparison with blood meal results in the present study. It is noteworthy that results of avian surveys (via checklists) could vary depending upon the detectability of songs, habitat, time of day, weather condition, observer's skills, and other behavioral characteristics. It is likely that some "secretive" birds present at a given site may be underestimated or entirely overlooked. It is also worthy to note that detection of a certain species at a site during the day does not guarantee the species would be accessible to host-seeking mosquitoes at dusk/nighttime. Similarly, differences in bird behavior and other spatial and temporal factors may create conditions that some bird species become more suitable/accessible as hosts for mosquitoes.

Results

Blood meal analysis

Blood meal sources were successfully identified by DNA sequencing from 554 *Cs. melanura* and *Cs. morsitans* (Tables 2 and 3). Of the 529 *Cs. melanura* analyzed, 523 (98.9%) contained avian blood and 6 (1.1%) had mammalian blood. Of the 25 *Cs. morsitans* analyzed, 24 (96.0%) contained avian blood and 1 (4.0%) had mammalian blood.

We identified 55 different species of avian hosts for *Cs. melanura* (Table 2). The greatest preponderance of blood meals

Table 1. Estimated Frequencies of 43 Avian Species in Descending Order, from Most to Least Frequently Observed from June Through November

Species common name	Species scientific name	Family (order)	Avgerage monthly frequencey						
			Jun	Jul	Aug	Sept	Oct	Nov	Average
American Robin	*Turdus migratorius*	Turdidae (Passeriformes)	0.720	0.644	0.481	0.590	0.541	0.381	0.559
Black-capped Chickadee	*Poecile atricapillus*	Paridae (Passeriformes)	0.493	0.454	0.416	0.652	0.659	0.571	0.541
Blue Jay	*Cyanocitta cristata*	Corvidae (Passeriformes)	0.516	0.410	0.373	0.667	0.694	0.506	0.528
American Goldfinch	*Carduelis tristis*	Fringillidae (Passeriformes)	0.614	0.610	0.558	0.527	0.430	0.418	0.526
Mourning Dove	*Zenaida macroura*	Columbidae (Columbiformes)	0.625	0.546	0.502	0.553	0.465	0.367	0.510
Song Sparrow	*Melospiza melodia*	Emberizidae (Passeriformes)	0.573	0.536	0.407	0.496	0.539	0.314	0.477
Northern Cardinal	*Cardinalis cardinalis*	Cardinalidae (Passeriformes)	0.534	0.495	0.385	0.507	0.492	0.426	0.473
American Crow	*Corvus brachyrhynchos*	Corvidae (Passeriformes)	0.476	0.346	0.390	0.515	0.570	0.506	0.467
Gray Catbird	*Dumetella carolinensis*	Mimidae (Passeriformes)	0.662	0.518	0.487	0.614	0.265	0.033	0.430
Downy Woodpecker	*Picoides pubescens*	Picidae (Piciformes)	0.356	0.364	0.301	0.497	0.513	0.390	0.404
Tufted Titmouse	*Baeolophus bicolor*	Paridae (Passeriformes)	0.426	0.357	0.229	0.352	0.414	0.354	0.355
House Sparrow	*Passer domesticus*	Passeridae (Passeriformes)	0.407	0.366	0.340	0.345	0.304	0.295	0.343
European Starling	*Sturnus vulgaris*	Sturnidae (Passeriformes)	0.388	0.303	0.332	0.365	0.327	0.285	0.333
Common Grackle	*Quiscalus quiscula*	Icteridae (Passeriformes)	0.626	0.478	0.309	0.291	0.230	0.064	0.333
White-breasted Nuthatch	*Sitta carolinensis*	Sittidae (Passeriformes)	0.299	0.307	0.206	0.350	0.379	0.337	0.313
Cedar Waxwing	*Bombycilla cedrorum*	Bombycillidae (Passeriformes)	0.440	0.353	0.381	0.396	0.131	0.090	0.298
Red-winged Blackbird	*Agelaius phoeniceus*	Icteridae (Passeriformes)	0.636	0.502	0.208	0.077	0.195	0.081	0.283
Common Yellowthroat	*Geothlypis trichas*	Parulidae (Passeriformes)	0.470	0.340	0.176	0.302	0.142	0.009	0.240
House Finch	*Carpodacus mexicanus*	Fringillidae (Passeriformes)	0.252	0.210	0.184	0.203	0.322	0.240	0.235
Northern Flicker	*Colaptes auratus*	Picidae (Piciformes)	0.288	0.200	0.118	0.365	0.269	0.093	0.222
Chipping Sparrow	*Spizella passerina*	Emberizidae (Passeriformes)	0.374	0.303	0.121	0.151	0.191	0.028	0.195
Eastern Phoebe	*Sayornis phoebe*	Tyrannidae (Passeriformes)	0.253	0.174	0.172	0.324	0.229	0.004	0.193
Baltimore Oriole	*Icterus galbula*	Icteridae (Passeriformes)	0.503	0.218	0.205	0.062	0.028	0.005	0.170
Yellow Warbler	*Dendroica petechia*	Parulidae (Passeriformes)	0.476	0.252	0.174	0.081	0.004	0.001	0.165
Eastern Towhee	*Pipilo erythrophthalmus*	Emberizidae (Passeriformes)	0.301	0.224	0.150	0.168	0.101	0.017	0.160
Red-eyed Vireo	*Vireo olivaceus*	Vireonidae (Passeriformes)	0.324	0.178	0.068	0.206	0.067	0.001	0.141
Yellow-rumped Warbler	*Setophaga coronata*	Parulidae (Passeriformes)	0.058	0.051	0.018	0.113	0.437	0.128	0.134
House Wren	*Troglodytes aedon*	Troglodytidae	0.280	0.169	0.094	0.162	0.050	0.002	0.126
Red-bellied Woodpecker	*Melanerpes carolinus*	Picidae (Piciformes)	0.140	0.093	0.038	0.087	0.215	0.141	0.119
Savannah Sparrow	*Passerculus sandwichensis*	Emberizidae (Passeriformes)	0.068	0.053	0.035	0.162	0.319	0.073	0.118
Brown-headed Cowbird	*Molothrus ater*	Icteridae (Passeriformes)	0.366	0.190	0.062	0.028	0.041	0.021	0.118
Eastern Wood-Pewee	*Contopus virens*	Tyrannidae (Passeriformes)	0.304	0.189	0.088	0.064	0.005	0.000	0.108
Warbling Vireo	*Vireo gilvus*	Vireonidae (Passeriformes)	0.212	0.100	0.048	0.107	0.001	0.000	0.078
Pine Warbler	*Dendroica pinus*	Parulidae (Passeriformes)	0.204	0.129	0.010	0.064	0.041	0.008	0.076
Great Crested Flycatcher	*Myiarchus crinitus*	Tyrannidae (Passeriformes)	0.259	0.132	0.047	0.014	0.001	0.000	0.076
Scarlet Tanager	*Piranga olivacea*	Cardinalidae (Passeriformes)	0.226	0.159	0.017	0.031	0.009	0.000	0.074
Rose-breasted Grosbeak	*Pheucticus ludovicianus*	Cardinalidae (Passeriformes)	0.206	0.119	0.063	0.044	0.006	0.002	0.073
Ovenbird	*Seiurus aurocapilla*	Parulidae (Passeriformes)	0.236	0.127	0.022	0.023	0.004	0.000	0.069
Wood Thrush	*Hylocichla mustelina*	Turdidae (Passeriformes)	0.241	0.139	0.018	0.009	0.002	0.000	0.068
Black-and-white Warbler	*Mniotilta varia*	Parulidae (Passeriformes)	0.142	0.059	0.035	0.109	0.023	0.002	0.062
Field Sparrow	*Spizella pusilla*	Emberizidae (Passeriformes)	0.110	0.058	0.024	0.040	0.082	0.019	0.055
Veery	*Catharus fuscescens*	Turdidae (Passeriformes)	0.186	0.101	0.008	0.015	0.000	0.000	0.052
Northern Waterthrush	*Parkesia noveboracensis*	Parulidae (Passeriformes)	0.054	0.015	0.027	0.036	0.006	0.000	0.023

Cells highlighted in gray indicate bird species that served as the source of blood meals for *Culiseta melanura.*

($n = 115$, 21.7%) was from American Robin, *Turdus migratorius* Linnaeus. Other frequent hosts included Tufted Titmouse, *Baeolophus bicolor* (L.) ($n = 46$, 8.7%); Black-capped Chickadee, *Poecile atricapillus* (L.) ($n = 45$, 8.5%); Scarlet Tanager, *Piranga olivacea* (Gmelin) ($n = 36$, 6.8%); and Field Sparrow, *Spizella pusilla* (Wilson) ($n = 33$, 6.2%). The 55 species were members of 8 avian orders and 25 families. The order Passeriformes constituted 97.0% ($n = 507$) of blood meals acquired by *Cs. melanura*, followed by Columbiformes 1.7% ($n = 9$), Cuculiformes 0.4% ($n = 2$), and 0.2% ($n = 1$) each of the Accipitriformes, Falconiformes, Gruiformes, Pelecaniformes, and Piciformes. Of 25 avian families, Turdidae (thrushes) with 34.6% ($n = 181$) of blood meals, Paridae (chickadees) with 17.4% ($n = 91$), and Cardinalidae (cardinals) with 14% ($n = 73$) were the 3 most frequent hosts.

Mammalian hosts for *Cs. melanura* were identified as white-tailed deer, *Odocoileus virginianus* Zimmermann ($n = 3$, 0.6%), domestic cow, *Bos taurus* L. ($n = 2$, 0.4%), and human, *Homo sapiens* L. ($n = 1$, 0.2%) (Table 2); 3 of these mosquitoes contained mixed blood meals of avian and mammalian origin. Of

Table 2. Number and Percentage of Avian- and Mammalian-derived Blood Meals Identified From *Culiseta melanura* Sampled in Massachusetts 2007–2008

			County						
Avian (common name)	*Scientific name*	*Residency Code*	*Bristol No. (%)*	*Essex No. (%)*	*Middlesex No. (%)*	*Norfolk No. (%)*	*Plymouth No. (%)*	*Worcester No. (%)*	*Total*
American Robin	*Turdus migratorius*	P, T	76 (27.2)	8 (8.9)	4 (15.4)	6 (17.6)	17 (20.2)	4 (25.0)	115
Tufted Titmouse	*Baeolophus bicolor*	P	32 (11.5)	5 (5.6)	2 (7.7)	2 (5.9)	3 (3.6)	2 (12.5)	46
Black-capped Chickadee	*Poecile atricapillus*	P	25 (9.0)	10 (11.1)	1 (3.8)	1 (2.9)	8 (9.5)		45
Scarlet Tanager	*Piranga olivacea*	S	7 (2.5)	4 (4.4)	3 (11.5)	1 (2.9)	21 (25.0)		36
Field Sparrow	*Spizella pusilla*	S	26 (9.3)	4 (4.4)	1 (3.8)		2 (2.4)		33
Northern Cardinal	*Cardinalis cardinalis*	P	5 (1.8)	17 (18.9)	2 (7.7)		6 (7.1)		30
Wood Thrush	*Hylocichla mustelina*	S	8 (2.9)	7 (7.8)		11 (32.3)	1 (1.2)		27
Red-eyed Vireo	*Vireo olivaceus*	S	12 (4.3)	1 (1.1)		1 (2.9)		1 (6.25)	15
Baltimore Oriole	*Icterus galbula*	S	2 (0.7)	4 (4.4)		6 (17.6)	1 (1.2)	1 (6.25)	14
Brown-headed Cowbird	*Molothrus ater*	P, T	10 (3.6)				1 (1.2)		11
Chipping Sparrow	*Spizella passerina*	S	9 (3.2)				1 (1.2)		10
Common Yellowthroat	*Geothlypis trichas*	S	4 (1.4)	1 (1.1)	2 (7.7)		1 (1.2)	1 (6.25)	9
Gray Catbird	*Dumetella carolinensis*	S	5 (1.8)			1 (2.9)	3 (3.6)		9
Mourning Dove	*Zenaida macroura*	P	3 (1.1)	4 (4.4)		2 (5.9)			9
Cedar Waxwing	*Bombycilla cedrorum*	P, T	5 (1.8)	2 (2.2)			1 (1.2)		8
Grasshopper Sparrow	*Ammodramus savannarum*	S		2 (2.2)	2 (7.7)	2 (5.9)	1 (1.2)		7
Red-winged Blackbird	*Agelaius phoeniceus*	P, T	5 (1.8)	1 (1.1)			1 (1.2)		7
Rose-breasted Grosbeak	*Pheucticus ludovicianus*	S	2 (0.7)	2 (2.2)	1 (3.8)	1 (2.9)		1 (6.25)	7
House Wren	*Troglodytes aedon*	S	3 (1.1)		1 (3.8)		2 (2.4)		6
Ovenbird	*Seiurus aurocapilla*	S	4 (1.4)		1 (3.8)		1 (1.2)		6
Black-and-white Warbler	*Mniotilta varia*	S	5 (1.8)						5
House Finch	*Carpodacus mexicanus*	P		4 (4.4)			1 (1.2)		5
Blue Jay	*Cyanocitta cristata*	P, T	1 (0.4)	2 (2.2)	1 (3.8)				4
Common Grackle	*Quiscalus quiscula*	P, T	1 (0.4)				3 (3.6)		4
Eastern Towhee	*Pipilo erythrophthalmus*	S	3 (1.1)				1 (1.2)		4
Pine Warbler	*Dendroica pinus*	S	3 (1.1)	1 (1.1)					4
Savannah Sparrow	*Passerculus sandwichensis*	S					2 (2.4)	1 (6.25)	3
Veery	*Catharus fuscescens*	S	3 (1.1)						3
Yellow Warbler	*Dendroica petechia*	S	2 (0.7)	1 (1.1)					3
Yellow-rumped Warbler	*Dendroica coronata*	P, T	2 (0.7)	1 (1.1)					3
Other Avian Species			15 (5.4)	7 (7.8)	5 (19.2)		3 (3.6)	5 (31.25)	35
Mammalian									
White-tailed Deer	*Odocoileus virginianus*		1 (0.4)[a]				2 (2.4)[b]		3
Cow	*Bos taurus*			2 (2.2)[c]					2
Human	*Homo sapiens*						1 (1.2)		1
Total			279	90	26	34	84	16	529

Residency codes: P, permanent resident (found year round in the state); S, summer resident (present in the state during the nesting season); T, transient (migrates through the state in spring and/or fall).

[a]Bristol County white-tailed deer sample contained mixed blood with a Black-capped Chickadee.

[b]White-tailed deer samples from Plymouth contained mixed blood with a Common Yellowthroat and a Black-capped Chickadee.

[c]One cow sample from Essex County contained mixed blood with a House Finch.

these, 1 specimen from Bristol County acquired a blood meal from a Black-capped Chickadee and white-tailed deer; 1 specimen from Plymouth County was identified with mixed blood from Common Yellowthroat, *Geothlypis trichas* (L.), and white-tailed deer; and 1 specimen from Essex County contained blood from a House Finch, *Carpodacus mexicanus* (Müller), and domestic cow.

The composition of 25 avian- and mammalian-derived blood meals for *Cs. morsitans* is shown in Table 3. We identified 14 avian species as hosts for *Cs. morsitans*. The largest number of blood meals was from Wood Thrush, *Hylocichla mustelina* (Gmelin) ($n = 7$, 28.0%), followed by Tufted Titmouse ($n = 3$, 12.0%), Black-capped Chickadee, Eastern Towhee, *Pipilo erythrophthalmus* (L.) ($n = 2$, 8.0% each), and 10 other avian species ($n = 1$, 4.0% each). White-tailed deer was the only mammalian host for *Cs. morsitans* ($n = 1$, 4.0%).

Virus isolations from blood-fed mosquitoes

All blood-fed mosquitoes were tested for infection with EEE virus and/or other arboviruses that currently circulate in the region. Four virus isolates were recovered from the head and thorax of individual blood-fed *Cs. melanura* mosquitoes in Vero cell culture, and later were identified as EEE, West Nile, and Highland J viruses by real-time RT-PCR assays, suggesting disseminated infection. The identity of viruses, trap type, dates of collection, location, and blood meal sources for these mosquitoes are provided in Table 4.

Table 3. Number and Percentage of Avian- and Mammalian-Derived Blood Meals Identified From *Culiseta morsitans* Sampled in Massachusetts 2007–2008

			County						
Avian (common name)	*Scientific name*	*Residency code*	*Bristol No. (%)*	*Essex No. (%)*	*Middlesex No. (%)*	*Norfolk No. (%)*	*Plymouth No. (%)*	*Worcester No. (%)*	*Total*
Wood Thrush	*Hylocichla mustelina*	S		7 (30.4)					7
Tufted Titmouse	*Baeolophus bicolor*	P		3 (13.0)					3
Black-capped Chickadee	*Parus atricapillus*	P		2 (8.7)					2
Eastern Towhee	*Pipilo erythrophthalmus*	S		2 (8.7)					2
Brown-headed Cowbird	*Molothrus ater*	P, T		1 (4.3)					1
Cedar Waxwing	*Bombycilla cedrorum*	P, T		1 (4.3)					1
Common Yellowthroat	*Geothlypis trichas*	S		1 (4.3)					1
Cooper's Hawk	*Accipiter cooperi*	P		1 (4.3)					1
Field Sparrow	*Spizella pusilla*	S		1 (4.3)					1
Gray Catbird	*Dumetella carolinensis*	S		1 (4.3)					1
Mourning Dove	*Zenaida macroura*	P		1 (4.3)					1
Red-eyed Vireo	*Vireo olivaceus*	S		1 (4.3)					1
Scarlet Tanager	*Piranga olivacea*	S		1 (4.3)					1
American Robin	*Turdus migratorius*	P, T						1 (100)	1
Mammalian									
White-tailed Deer	*Odocoileus virginianus*		1 (100)						1
Total			1	23				1	25

Residency codes: P, permanent resident (found year round in the state); S, summer resident (present in the state during the nesting season); T, transient (migrates through the state in spring and/or fall).

Avian frequency analysis

Residency status of the bird species that frequently served as the source of blood meals for *Cs. melanura* is shown in Table 2. Of these, six species were permanent residents (found year round), 18 were summer residents (present during the nesting season), and seven were permanent residents with part of the population transient in the spring and/or fall.

Estimated frequency patterns of bird occurrence in the 6 counties were similar and thus were combined for analysis (Table 1, Fig. 2). We focused on 43 of more than 330 bird species reported from the region as some of the most commonly encountered birds that reside in swamp localities and share habitats with *Cs. melanura* or reside in adjacent swamp-border areas frequented by host-seeking females. American Robin, Tufted Titmouse, Black-capped Chickadee, Northern Cardinal, *Cardinalis cardinalis* (L.), Gray Catbird, *Dumetella carolinensis* (L.), and Mourning Dove, *Zenaida macroura* (L.) together constituted 48.5% ($n = 254$) of all avian-derived blood meals by *Cs. melanura*. These birds were also among the most frequently encountered avian species in the region based on the eBird database. Blue Jay, *Cyanocitta cristatai* (L.), was identified as the 3rd most common bird species in the region, but constituted only 0.8% ($n = 4$) of avian-derived blood meals by *Cs. melanura.* Although American Crow, *Corvus brachyrhynchos* Brehm, was the 8th most frequent bird species in the region, we did not identify a single crow–derived blood meal in either *Cs. melanura* or *Cs. morsitans*. Similarly, no *Cs. melanura* specimens were identified with blood meals from American Goldfinch, *Carduelis tristis* (L.) or Downy Woodpecker, *Picoides pubescens* (L.), 2 comparatively abundant bird species in the region. Northern Waterthrush, *Parkesia noveboracensis* (Gmelin); Veery, *Catharus fuscescens* (Stephens); and Field Sparrow were among the bird species that had relatively low frequencies. There were 10 bird species with relatively high frequencies in the study sites (Table 1, Fig. 2); however, these birds were not identified as hosts for *Cs. melanura*.

Discussion

Our study provides insight into the vector–host interactions of *Cs. melanura* and *Cs. morsitans* in Massachusetts, an area in the northeastern United States with multiple EEE virus foci. Our results confirm the strong ornithophilic nature of *Cs.*

Table 4. Identity of Virus Isolates from Individual Blood-Fed *Culiseta melanura* Mosquitoes, Date of Collection, Location, Trap Type, and Blood Meal Sources

Date of collection	*Location*	*Trap Type*	*Virus isolate*	*Blood meals source*
9/3/2007	Peterson Swamp, Halifax, Plymouth County	Resting box	Eastern equine encephalitis virus	—
9/9/2007	Pine Swamp, Raynham, Bristol County	Miniature light trap	West Nile virus	—
9/20/2007	Hopkinton Road, Westborough, Worcester County	Resting box	Highland J virus	American Robin (*Turdus migratorius*)
9/5/2008	Peterson Swamp, Halifax, Plymouth County	Resting box	Highland J virus	Scarlet Tanager (*Piranga olivacea*)

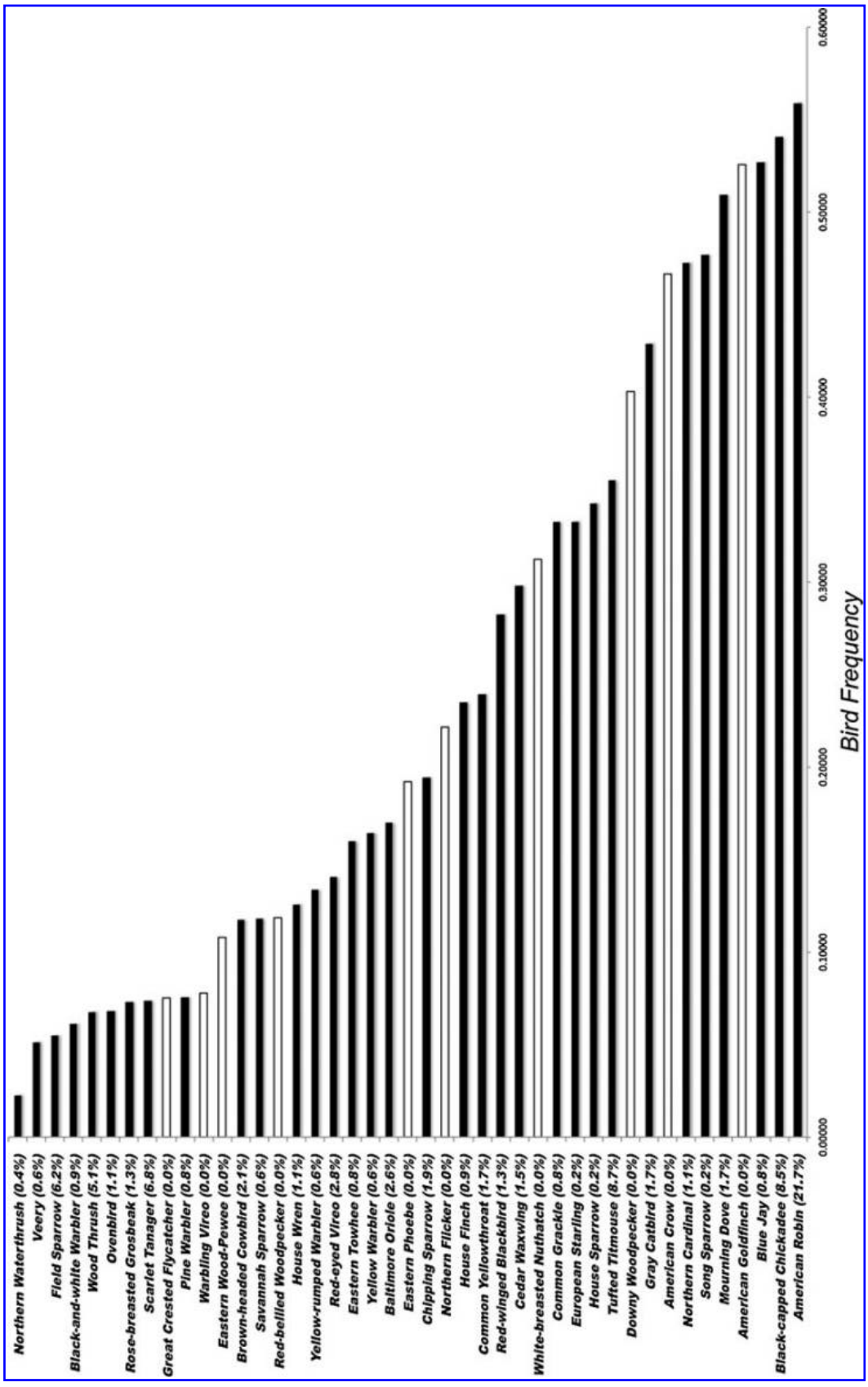

FIG. 2. Average estimated frequencies (June through November) of 43 avian species in Massachusetts. Bars highlighted in black indicate frequent avian species that also served as hosts for *Culiseta melanura*. Bars highlighted in white depict bird species that were not identified as sources of blood meals for *Culiseta melanura*. Percentages following species' names show the proportion of blood meals for *Cs. melanura* from each bird species.

melanura (99%) and *Cs. morsitans* (96%), in acquiring blood meals from avian hosts; however, we were also able to document, albeit low, that both of these species also acquire mammalian blood meals. Of note, was the identification of 1 human blood meal from *Cs. melanura*. This finding is particularly relevant to public health surveillance of EEE virus in Massachusetts, especially during the peak season of *Cs. melanura* abundance and coincident detection of the virus.

During the last several years, EEE virus has been isolated from 16 different mosquito species collected throughout northeastern United States (ArboNET; Centers for Disease Control and Prevention, Atlanta, GA). The overwhelming majority of isolations have been made from *Cs. melanura*, reaffirming its direct involvement in enzootic amplification of the virus within virus foci. Although EEE virus frequently infects *Cs. melanura*, this species has generally been considered an unlikely "bridge" vector because it feeds mainly on birds (Scott and Weaver 1989, Komar and Spielman 1994). However, in our study, a small proportion of *Cs. melanura* and *Cs. morsitans* acquired blood meals from mammalian hosts, including a human-derived blood meal from *Cs. melanura*.

Identification of mammalian-derived blood meals from *Cs. melanura* in the present study is consistent with the results of our earlier investigations on the feeding behavior of this mosquito species collected from 2 sites associated with an EEE virus focus in central New York (Molaei et al. 2006a) and from Connecticut (Molaei and Andreadis 2006). In New York, 0.8% and 5.0% ($n = 484$) of the blood meals for *Cs. melanura* were identified from mammals solely or in mixed blood meals, respectively. Similarly, in Connecticut 4.2% ($n = 48$) of the blood meals originated from mammals including equines. Thus identification of one human-derived blood meal from this mosquito species in the present study is credible based on human population density as one of the most abundant mammalian species in the region. The possibility of a potential contamination during laboratory analyses and PCR cannot be entirely ruled out; however, we are reasonably confident that experimentations have been carefully conducted, and that *Cs. melanura* occasionally feeds on mammals including humans. A recent study from New Jersey also documented 2 human-derived blood meals of 6 specimens with nonavian hosts (Apperson et al. 2004).

Earlier analysis of engorged *Cs. melanura*, collected from the perimeter of 2 red maple-white cedar freshwater swamps in the Taunton River basin in the towns of Easton and Raynham Massachusetts by using the precipitin technique, reported 0.5% ($n = 5$) mammalian blood-feeding in mosquitoes collected from the swamp perimeter in 1977 (Nasci and Edman 1981). Similarly, 0.3% ($n = 5$) and 0.4% ($n = 2$) of *Cs. melanura* were identified with mammalian-derived blood meals in mosquitoes collected from the outside swamp and perimeter, respectively, in 1978 (Nasci and Edman 1981). Mammalian species that served as the source of blood meals in the latter study included dog, rabbit, and cat. In the present study, we identified white-tailed deer, cow, and human as hosts for *Cs. melanura*. Although the rather small percentage of mammalian-derived blood meals does not permit a comprehensive discussion, differences are likely due to variations in collection methods, and abundance of the host species in the 2 studies. In a recent study in Connecticut, *Cs. melanura* was the predominant source of EEE virus (83 [68%] of 122 virus isolations) and the only species to support consistently high virus titers required for efficient transmission (Armstrong and Andreadis 2010). In retrospect, it is possible that a single EEE virus detection from 554 blood meals may be somewhat an underrepresentation of actual infection of mosquitoes during the study period. During the 2007 and 2008, rates of positive pools detected at these same sites in Massachusetts were 1.4% (25/1726) in 2007 and 1.8% (11/600) in 2008 tested by RT-PCR. These findings further implicate *Cs. melanura* as a potential "bridge" vector of EEE virus to humans and other mammals throughout the range of distribution, in addition to its prominent role as an enzootic vector.

Enzootic transmission of EEE virus depends on the frequent interaction of *Cs. melanura* with key virus-competent bird species, some of which may serve as "superspreaders." In the present study, 97% of all avian-derived blood meals originated from Passeriformes birds, including American Robin, Tufted Titmouse, Black-capped Chickadee, Scarlet Tanager, Field Sparrow, and other avian species possibly serving as reservoirs for EEE virus. We identified American Robin as the most frequent source of blood ($n = 115$, 21.7%) for *Cs. melanura*. Similarly, American Robins also served as a frequent source of blood for host-seeking *Cs. melanura* in neighboring Connecticut (22.9%, $n = 11$) (Molaei and Andreadis 2006) and New York (9.1%, $n = 46$) (Molaei et al. 2006a), and for *Culex* spp. mosquitoes throughout the Northeast and other regions of the United States (Apperson et al. 2002, Apperson et al. 2004, Molaei et al. 2006b, Savage et al. 2007, Hamer et al. 2009), thus implicating these birds as important amplifying hosts in enzootic transmission of arboviruses, including EEE and West Nile viruses.

American Robins are found throughout most of North America. Permanent and transient populations of this species use a wide variety of open and forested habitats in urban/suburban and rural settings, and active in riparian forests, early successional forests (Martin 1973, Hutto 1995, Sallabanks 1995), closed canopy forests, and woodlands (www.epa.gov/region1/ge/thesite/restofriver/reports/final_era/B%20%20Focus%Species%20Profiles/EcoRiskProfile_american_robin.pdf). American Robins are the most prominent tree-roosting birds in woodland habitats, where large flocks of these birds roost in summer months after nesting ends (Poole 2005). This creates a spatial overlap with activities of *Cs. melanura* in EEE virus foci throughout the region. Dates of the first clutch of American Robins in early April through late July in southern regions of the Northeast, and early May through early July in northern areas (*e.g.* northern Maine; http://goo.gl/1GIDB/) overlap temporally with the emergence of the 1st generation of *Cs. melanura*. American Robins are competent amplifying hosts for EEE virus based on the intensity of infection and duration of viremias after infection by mosquitoes (Komar et al. 1999). EEE virus also has been isolated from American Robins in Massachusetts (Main et al. 1988) and New Jersey (Crans et al. 1994). Moreover, antibody prevalence studies indicate that these birds are frequently exposed to EEE virus throughout the region (Dalrymple et al. 1972, Bast et al. 1973, Morris et al. 1975, Main et al. 1988, Crans et al. 1994, Gettman, personal communication). Our results further suggest that American Robins may play a more important role in early season virus amplification, due to their abundance and greater availability as hosts at this time of the year.

Tufted Titmouse and Black-capped Chickadee were the 2nd and 3rd most frequent hosts and together constituted 17.4% ($n = 91$) of blood meals for *Cs. melanura*. The Tufted Titmouse

is found throughout the eastern United States, and the Black-capped Chickadee is common in the east. Our estimated bird frequency data analysis also indicated that these two species are common in eastern Massachusetts (Table 1, Fig. 2). The preferred habitats for Tufted Titmouse and Black-capped Chickadee include deciduous and mixed deciduous/coniferous woodlands, open woods, swamps, and dense canopies (http://bna.birds.cornell.edu/). These birds are more common near edges of wooded areas, but can be found in the middle of large wooded tracts that make them accessible to host-seeking *Cs. melanura* and other woodland inhabiting mosquitoes. Tufted Titmouse and Black-capped Chickadee captured within EEE virus foci in Alabama (Stamm 1968), Maryland (Dalrymple et al. 1972), New Jersey (Crans et al. 1994), New York (Howard et al. 2004), Massachusetts (Main et al. 1988), and Michigan (McLean et al. 1985) have been reported to have a very high antibody prevalence.

Wood Thrushes were identified as a relatively frequent source of blood for *Cs. melanura* (5.1%, $n=27$) and as the most frequent source of blood for *Cs. morsitans* (28%, $n=7$), although the sample size for the latter mosquito species was small in the present study. Similarly, in earlier studies, the Wood Thrush served as the most frequent host for *Cs. melanura* (23.6%, $n=120$) and *Cs. morsitans* (30.9%, $n=42$) in New York (Molaei et al. 2006a), and as the 2nd most frequent host for *Cs. melanura* (12.5%, $n=6$) in Connecticut (Molaei and Andreadis 2006). Wood Thrushes breed in deciduous and mixed forests in the eastern United States where there are large trees, moderate understory, shade, and abundant leaf litter for foraging. The breeding range for these birds extends from Manitoba, Ontario, and Nova Scotia in southern Canada to northern Florida and from the Atlantic coast to the Missouri River and the eastern Great Plains (Farrand 1987). Frequent infection of Wood Thrushes with EEE virus has been reported. Of 42 isolations of EEE virus from more than 3000 birds bled in south Alabama, there were more from Wood Thrush than any other bird species (Stamm 1968). In New Jersey, early-season virus isolation from Wood Thrush and a few other bird species has been reported as evidence of a cryptic EEE virus cycle (Crans et al. 1994). In Massachusetts, Wood Thrush had the highest EEE virus antibody prevalence rates (26.7%) followed by Swamp Sparrow, *Melospiza georgiana* (Latham) (24.5%), American Robin (20.9%), and Ovenbird, *Seiurus aurocapilla* (L.) (18.2%) (Main et al. 1988).

We identified several other avian species as the sources of blood for *Cs. melanura* and *Cs. morsitans* at moderate or low frequency. Our estimated bird frequency data analyses also demonstrate that these species occur with varying degrees of frequency, but due to the microhabitat differences, they may not have been accessible to host-seeking mosquitoes. Nonetheless, a number of these species, most notably Scarlet Tanager; Northern Cardinal; Red-eyed Vireo, *Vireo olivaceus* (L.); Baltimore Oriole, *Icterus galbula* (L.); Common Yellowthroat, *Geothlypis trichas* (L.); Red-winged Blackbird, *Agelaius phoeniceus* (L.); House Wren, *Troglodytes aedon* (Vieillot); Ovenbird; Blue Jay; Common Grackle, *Quiscalus quiscula* (L.); and Veery have been shown to have high antibody prevalence for EEE virus, or virus has been isolated from these birds in New Jersey, New York, and Massachusetts (Emord and Morris 1984, Main et al. 1988, Crans et al. 1994, Komar and Spielman 1994, Howard et al. 2004). These reservoir-competent birds have been incriminated in the maintenance and amplification of EEE virus in the region (Komar et al. 1999).

To better understand vector–host interactions, and gain insight into the role of key bird species in EEE virus transmission, we examined estimated bird frequency data in the study region. We compared bird frequency data in the 6 counties from which engorged-mosquitoes were collected to each other and to the entire region, and because there were no appreciable differences, we used data for the entire region in our analyses. We identified more than 330 bird species as permanent resident, summer resident, and/or transient (birds that migrate through the region in spring and/or fall), with varying frequencies and abundance. Among 43 avian species identified as frequent birds in the region, 33 species also served as hosts for *Cs. melanura*, indicating that these mosquitoes acquire blood meals from the most common bird species in the region. However, there were a number of frequently observed species, including American Goldfinch; Downy Woodpecker; and White-breasted Nuthatch, *Sitta carolinensis* Latham that were not identified as the source of blood meals for *Cs. melanura* and *Cs. morsitans* in our analyses. The lack of feedings on these birds might indicate that local populations of *Cs. melanura* and *Cs. morsitans* are not attracted to these species. However, a more likely explanation is that there was no temporal and/or spatial overlap between mosquito and bird activities. Certain birds, such as Woodpeckers, spend the night deep inside tree holes and thus are isolated from host-seeking mosquitoes (Edman et al. 1972). Defensive behavior that prevents or interrupts mosquito blood feedings could also be considered as a contributing factor to the lack of blood feedings on some bird species as has been previously reported for European Starling, *Sturnus vulgaris* L. (Edman and Kale 1971, Hodgson et al. 2001).

Bridging transmission of arboviruses by mosquitoes would require flexibility in the phenotype, such that an earlier blood feeding on birds (virus-amplifying hosts) follows a later feeding on mammals particularly humans. Because of the predominately ornitophilic nature of *Cs. melanura* in blood feeding, there was little expectation of seasonal shifts from avian to mammalian hosts. However, a seasonal shift from American Robin to other avian species was noted. The chi-squared tests for linear trend showed that the proportion of American Robin–derived blood meals was significantly higher earlier in the season ($p<0.0001$); 37.3% and 22.6% of all avian-derived blood meals in June and July, respectively, were from this avian species (Table 5). This was during the time when blood-feeding activity of the 1st generation of *Cs. melanura* temporally overlapped with the influx of migrating American Robins and start of the breeding season throughout the region. However, blood feeding on American Robin decreased gradually as the season progressed. In August and September, 14.1% and 7.7% of the blood meals were from this species, respectively, and by October it further declined to 4.8%. Toward the end of the season, a variety of other avian species such as Tufted Titmouse, Black-capped Chickadees, Scarlet Tanager, and Field Sparrow more frequently served as hosts for *Cs. melanura* (Table 5). An earlier study on the blood-feeding pattern of *Cs. melanura* in Massachusetts also reported a trend toward increasing diversity as the season progressed (Nasci and Edman 1981). During spring and early summer, passerine birds were among the most frequent source of blood meal for *Cs. melanura*, whereas later in the season,

Table 5. Monthly Prevalence of Avian-Derived Blood Meals for *Culiseta melanura* in Massachusetts 2007–2008

Avian species	*June* (n = 153)[a] %	*July* (n = 159)[a] %	*August* (n = 99)[a] %	*September* (n = 91)[a] %	*October* (n = 21)[a] %
American Robin	37.3	22.6	14.1	7.7	4.8
Tufted Titmouse	4.6	10.7	12.1	9.9	4.8
Black-capped Chickadee	7.8	7.5	6.1	15.4	4.8
Scarlet Tanager	5.2	3.1	1	18.7	23.8
Field Sparrow	1.3	7.5	8.1	9.9	9.5
Northern Cardinal	1.3	9.4	7.1	4.4	9.5
Wood Thrush	3.3	6.3	10.1	2.2	0
Other avian species	39.2	32.7	41.4	31.9	42.9

[a]Indicates total number of blood meals from various avian species in each month.

nonpasserines and nonavian hosts were used, and feeding on mammalian hosts peaked during the 1st week of September (Nasci and Edman 1981).

In the present study, a relatively small percentage of *Cs. melanura* was identified with blood meals from certain avian species, despite our efforts for collecting an increased number of engorged mosquitoes by using numerous sites and 2 trap types. This limitation in conjunction with the scarcity of critical and more recent information on the community structure, breeding, and daily activities of various avian species precludes a comprehensive discussion on the potential shift in host selection of *Cs. melanura*. Nonetheless, availability and abundance of various vertebrate species may influence temporal heterogeneity in the host-feeding pattern of mosquitoes and potential shifts from avian to mammalian hosts or from a certain members of avian community to the others (Molaei et al. 2006b, Kilpatrick et al. 2007, Hamer et al. 2008). Other factors, such as increased mosquito abundance, physiological changes in mosquito host preference, and defensive behavior in birds, also have been postulated as underlying causes for variations in seasonal blood-feedings patterns (Tempelis et al. 1965, Edman et al. 1974, Nelson et al. 1976, Thiemann et al. 2011).

Although the focus of the present study was on the role of *Cs. melanura* and *Cs. morsitans*, it is also important to discuss the contribution of other potential "bridge" vectors in the genera of *Culex*, *Aedes*, *Ochlerotatus*, *Anopheles*, and *Coquillettidia* to epidemic/epizootic transmission of EEE virus in Massachusetts and other regions of the Northeast. EEE virus has been isolated from these mosquitoes in Connecticut (Andreadis et al. 1998), and throughout northeastern United States (Crans and Schulze 1986, Centers for Disease Control and Prevention 2006). Biting risk of potential epidemic/epizootic mosquito vectors in EEE virus foci in Bristol and Plymouth Counties in southeastern Massachusetts, where human and horse cases have been historically reported, was estimated using carbon dioxide–baited light traps for capturing adult mosquitoes (Moncayo and Edman 1999). It has been suggested that *Cq. perturbans*, *Oc. canadensis*, and *Culex salinarius* Coquillett were more likely vectors of EEE virus in Massachusetts than *Ae. vexans*, *Anopheles punctipennis* (Say), and *Anopheles quadrimaculatus* Say. Susceptibility to *per os* infection and potential salivary transmission for EEE virus in 6 mosquito species also have been investigated in an earlier study in Massachusetts (Vaidyanathan et a. 1997).

On the basis of estimates of laboratory vector competence, frequent EEE virus isolations from field-collected mosquitoes, distance from forest resting habitats during host seeking, blood-feeding patterns coinciding with human disease, and sufficient host diversity to act as "bridge" vectors from birds to mammals, mosquito species were ranked from the most to least probable epidemic vectors: *Cx. salinarius*, *An. quadrimaculatus*, *Oc. canadensis*, *Cq. perturbans*, *Ae. vexans*, and *An. punctipennis.* Of these, *Ae. vexans* and *An. punctipennis* were unable to transmit EEE virus under laboratory conditions (Merrill et al. 1934, Ten Broeck and Merrill 1935, Davis 1940, Chamberlain et al. 1954, Wallis and Main 1974). Comparatively larger number of isolations from *Cq. perturbans* collected in Massachusetts and New York, where the greatest number of human cases has been correspondingly reported, in concert with catholic feeding habits (Edman 1971, Magnarelli 1977, Apperson et al. 2002, 2004, Molaei et al. 2008), abundance, and vector competence, make this mosquito species a likely suspect involved in epidemic/epizootic transmission of EEE virus to humans and equines in Massachusetts and throughout the region.

In conclusion, our study further clarifies the host associations of *Cs. melanura* and *Cs. morsitans* in this region of the northeastern United States. We find that these mosquitoes feed primarily on birds and focus their feeding activity on several common Passeriformes species capable of supporting EEE virus transmission. A small proportion of *Cs. melanura* and *Cs. morsitans* acquired blood meals from mammalian hosts including humans, suggesting their potential involvement in epidemic/epizootic transmission of EEE virus to humans and equines.

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Author Disclosure Statement

No competing financial interests exist.

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