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Seasonal Patterns in Eastern Equine Encephalitis Virus Antibody in Songbirds in Southern Maine

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Abstract

The intent of this study was to assess passerine eastern equine encephalitis virus (EEEv) seroprevalence during the breeding season in southern Maine by testing songbird species identified in the literature as amplifying hosts of this virus. In 2013 and 2014, we collected serum samples from songbirds at a mainland site and an offshore island migratory stopover site, and screened samples for EEEv antibodies using plaque reduction neutralization tests. We compared seasonal changes in EEEv antibody seroprevalence in young (hatched in year of capture) and adult birds at the mainland site, and also compared early season seroprevalence in mainland versus offshore adult birds. EEEv seroprevalence did not differ significantly between years at either site. During the early season (May), EEEv antibody seroprevalence was substantially lower (9.6%) in the island migrant adults than in mainland adults (42.9%), 2013–2014. On the mainland, EEEv antibody seroprevalence in young birds increased from 12.9% in midseason (June–August) to 45.6% in late season (September/October), 2013–2014. Seroprevalence in adult birds did not differ between seasons (48.8% vs. 53.3%). EEEv activity in Maine has increased in the past decade as measured by increased virus detection in mosquitoes and veterinary cases. High EEEv seroprevalence in young birds—as compared to that of young birds in other studies—corresponded with two consecutive active EEEv years in Maine. We suggest that young, locally hatched songbirds be sampled as a part of long-term EEEv surveillance, and provide a list of suggested species to sample, including EEEv ''superspreaders.''

Keywords: *Culiseta melanura*, eastern equine encephalitis virus, enzootic, epizootic, songbirds

Introduction

Eastern equine encephalitis virus (EEEv) is a mosquito-borne alphavirus that causes disease in wildlife (Fothergill et al. 1938, Tyzzer et al. 1938, Hays et al. 1962, Schmitt et al. 2007), domestic livestock (Gilter and Shahan 1933), and humans (Webster and Wright 1938). Sporadic human cases have occurred across the eastern and Midwestern United States (CDC 2012) with high mortality and frequent serious morbidity and long-term sequelae (Ayres and Feemster 1949, Deresiewicz et al. 1997).

Outbreaks of human EEEv disease have been common in Massachusetts since 1938 (MDPH 2012). Among New England states, Vermont (Saxton-Shaw et al. 2015) and Maine (CDC 2012, 2014, CDC/USGS 2015) have the shortest histories of EEEv activity. Maine had its first veterinary EEEv outbreak in 2009 (Lubelczyk et al. 2013) and its first human case in early October, 2014 (CDC 2014). *Culiseta melanura* mosquitoes, the primary vector of EEEv, were 0% to 0.8% positive between 2001 (when testing began) and 2012, but tested 8.6% and 4.3% positive in 2013 and 2014, respectively (MECDC 2015). During 2008–2014,

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there were 25 veterinary deaths due to EEEv in 5 of 7 years (including 2013 and 2014), compared with only two cases in 1 year during 2001–2007 (Maine CDC).

C. melanura and avian hosts maintain EEEv in enzootic cycles (Armstrong and Andreadis 2010) in New England. Some species of birds and *C. melanura* are associated with freshwater wetland forest habitats (Moore et al. 1993) represented in Maine by red maple (*Acer rubrum*) and cedar (*Thuja*) swamps and spruce (*Picea*) bogs. *C. melanura* larvae overwinter in crypts, then the first adult generation emerges in late April into June, feeds on birds, then oviposits in crypts. As the second generation emerges June through August, EEEv can be isolated from both mosquitoes and birds (indicating transmission) and may increase (amplify) and remain elevated through August.

Many species of songbirds are competent reservoir hosts. Songbirds have been tested for antibody in at least six U.S. states (Stamm 1963, Dalrymple et al. 1972, Emord and Morris 1984, McLean et al. 1985, Main et al. 1988, Crans et al. 1994), but to our knowledge not in northern New England (Maine, New Hampshire, and Vermont). The present study was initiated because tracking EEEv in reservoir passerine hosts would improve our understanding of the role of birds in EEEv activity across and within years in Maine.

The intent of this study was to assess passerine EEEv seroprevalence in southern Maine by testing songbird species identified in the literature as amplifying hosts of this virus. The objective was to measure breeding season EEEv antibody seroprevalence in young (hatch year) and adult birds on a mainland site and an offshore migratory stopover island site during 2013 and 2014. To meet our objective, we collected serum samples from songbirds at two sites in southern Maine and screened them for EEEv antibodies using plaque reduction neutralization tests, and compared differences in seropositivity. Our main expectations were that (1) in mid-summer, seroprevalence in young (recently hatched) birds would be lower than in adult birds, because hatch-year birds would be relatively naive immunologically compared with adults, which could have been exposed in previous years, and that (2) seroprevalence in young birds would increase from mid to late summer, reflecting seasonal enzootic EEEv amplification.

Materials and Methods

Field collection

We collected serum samples from songbirds at two sites in southern Maine during the course of ongoing mist-netting and banding activities. At the Shoals Marine Laboratory, researchers have conducted banding since 1981 at Appledore Island Migration Station (42°59′12.4"N 70°36′51.3"W), an offshore migratory bird stopover site. The Biodiversity Research Institute has conducted banding since 2011 at the River Point Conservation Area (43°44′1.6"N 70°17′30.6"W), a mainland stopover and breeding site in Falmouth. Birds were banded under USGS Federal Bird Banding Permit 22636 (mainland) or 22243 (island). During the course of the research activities at these sites, we collected blood samples from species, including Veery (*Catharus fuscescens*), Hermit Thrush (*Catharus guttatus*), American Robin (*Turdus migratorius*), Gray Catbird (*Dumetella carolinensis*), Ovenbird (*Seiurus aurocapilla*), Northern Waterthrush (*Parkesia noveboracensis*), Common Yellowthroat (*Geothlypis trichas*), White-throated Sparrow (*Zonotrichia albicollis*), Song Sparrow (*Melospiza melodia*), and Northern Cardinal (*Cardinalis cardinalis*). These were species commonly EEEv seropositive in other studies (Stamm 1963, Dalrymple et al. 1972, Emord and Morris 1984, McLean et al. 1985, Main et al. 1988, and Crans et al. 1994).

''Adult'' designated a bird hatched in a year before the year of capture, and ''young'' designated birds hatched in the year of their capture. Island adult birds were captured May 16 through June 2, 2013 and May 14 through June 2, 2014. Mainland birds were captured May 5 through October 26, 2013 and May 13 through October 31, 2014. Young birds were captured at the mainland site July 3 through October 26, 2013 and July 1 through October 31, 2014.

In the field, researchers determined sex, fat condition (fat score in the tracheal pit recorded on a 0–7 scale), and breeding condition determined by the presence or absence of a brood patch in females or cloacal protuberance in males (Gosler et al. 1998, Desante et al. 2015). After banding the birds, researchers drew $\leq 50\,\mu$L of blood, from the brachial vein, into 75-mm heparinized Mylar-coated hematocrit tubes. Tubes were centrifuged at 11,000 rpm for 5 min and the serum fraction delivered into BD Microtainers® (Becton, Dickinson and Company, Franklin Lakes, NJ), which were shipped to the U.S. Centers for Disease Control and Prevention in Fort Collins, Colorado for testing.

Laboratory testing

Bird serum samples were diluted 1:10 and screened for EEEv-neutralizing antibodies by plaque-reduction neutralization assay. Any neutralizing specimens were retested and titrated for confirmation. Serum samples were considered positive for EEEv antibodies if they neutralized 80% of a challenge dose of ≈ 100 plaque-forming units of Sindbis-EEE chimeric virus as previously described by Wang et al. (2007) and Johnson et al. (2011), with no modifications to their methods. Dilutions were 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640 (*i.e.*, 20 was the lowest titer and 640 the highest).

Analysis

The pattern of EEEv activity (MDPH 2012) can be divided into three seasons: early (April to June, first generation of *C. melanura*), middle (June/July/August, second generation *C. melanura*, enzootic transmission/amplification) and late (September/October, transmission/continued high infection in mosquitoes until hard frosts). We divided the bird blood samples into three periods to correspond with three EEEv activity seasons: ''early'': May 3 through June 2; ''middle,'' June 3 through August 31, and ''late,'' September 1 through October 31. The June 2 cut point aligned early season island and mainland samples from adult birds.

We tabulated seroprevalence (percent EEEv seropositive) by site, year, season, and age group (pooling across species). We then tested for differences among groups by implementing two logistic regression models:

1. Mainland EEEv seroprevalence as a function of age + season + year + two-way + three-way interactions, where age = adult or young, season = middle or late, and year = 2013 or 2014 (there were no young birds in the early part of the breeding season). The comparisons of interest were seasonal differences for each age

group, and age group differences for each season, accounting for annual differences, if any.

2. Early season, adult bird EEEv seroprevalence as a function of site + year + two-way interaction, where site = island or mainland, and year = 2013 or 2014). The comparison of interest was island versus mainland, accounting for annual differences, if any.

We used the SAS/STAT® (Version 9.3, SAS Institute, Inc., Cary, NC) logistic procedure with a binomial response (positive for EEEv antibody = 1, negative = 0). Within proc logistic we used the glm parameterization option in the class statement, and selected the ilink and difference options in the lsmeans statement. Least-squares means in a logistic regression are estimates of the linear predictors on the logit scale and ilink applies the inverse-link transformation to obtain event probabilities, that is, the proportion EEEv antibody-positive for each comparison group (SAS Institute Inc. 2011). The difference option tested for differences in proportions among comparison groups. We specified a Bonferroni adjustment for multiple comparisons. We accepted overall model significance at Wald chi-square $p \leq 0.05$, eliminated interaction terms where Wald chi-square $p > 0.10$, and rejected the null hypothesis of no differences in proportions (EEEv seroprevalence) where adjusted Z-value $p \leq 0.05$. Sample year was not significant in either model and not discussed further. We tabulated EEEv test results by species, but sample size was not robust enough to model EEEv seroprevalence differences at the species level.

Previous studies tested for an association between EEEv seroprevalence and sex, fat condition, and breeding condition in adult birds; we tested for these associations for each year for the mainland adults, using one Fischer's exact test each for sex, fat condition, and breeding condition (pooled across years and seasons).

Results

Model 1 (Mainland young and adult birds): In young birds, EEEv seroprevalence (years combined) was lower in midseason (June–August) than late season (September/October) (12.9% vs. 45.6%, $Z = -2.90$, $p = 0.02$, Table 1). In contrast, in mainland adults there were no differences in EEEv seroprevalence between the middle and late seasons (48.8% vs. 53.3%, $Z = -0.30$, $p = 1.00$). Midseason EEEv seroprevalence in young birds was lower than in adults (12.9% vs. 48.8%, $Z = -3.00$, $p = 0.02$, Table 1). In late season, young and adult EEEv seroprevalence did not differ (45.6% vs. 48.8%, $Z = -0.30$, $p = 1.00$). Model 2 (early season island vs. mainland adult birds): In early season (May, years combined) EEEv antibody seroprevalence was substantially lower in the island migrants than in mainland adults (9.6% vs. 42.9%, $Z = -2.97$, $p = 0.003$, Table 1).

There were no associations between EEEv seroprevalence in adult songbirds and sex, fat condition, and breeding condition. Avian species composition and sample size of species differed among groups (island adult, mainland adult, mainland young (Table 2).

Discussion

The increase in EEEv antibody seroprevalence in young songbirds from midseason (June–August) to late season (September/October) was commensurate with generally understood patterns of EEEv amplification through the bird–mosquito transmission cycle, and has been observed consistently across studies (Dalrymple et al. 1972, Emord and Morris 1984, Crans et al. 1994). Although Kissling et al. (1954) found that in domestic pigeons (*Columba livia*) EEEv antibodies were maternally transferred to 30% of offspring, antibodies were barely detectable 4 weeks after hatching. Crans et al. (1994) reasoned that young birds reflected seasonal EEEv activity rather than maternally conferred immunity. This supports the premise that within-season EEEv activity was well represented by young songbirds in this study. However, late-season young birds disperse to and from various directions (Anders et al. 1998, Bayne and Hobson 2001, Mitchell et al. 2009) and, therefore, future work should focus on young birds known to have hatched locally.

In contrast to young birds, EEEv antibody prevalence of adult songbirds has been more complex to interpret because of EEEv exposure in prior years and variation in seroconversion and reversion (*e.g.*, Emord and Morris 1984). Appledore Island passage migrants may have reflected the

Table 1. Eastern Equine Encephalitis Virus Antibody Seroprevalence in Passerines Captured at Maine Banding Stations 2013–2014

			Tested			*Seroprevalence*					
						2013		*2014*		*Total*	
Site	*Age*[a]	*Season*	*2013*	*2014*	*Total*	*Positive*	*%*	*Positive*	*%*	*Positive*	*%*
Island Stopover	Adult	Early	41	42	83	6	14.6	2	4.8	8	9.6
Mainland	Adult	Early	10	4	14	4	40.0	2	50.0	6	42.9
		Middle	16	25	41	7	43.8	13	52.0	20	48.8
		Late	11	4	15	6	54.5	2	50.0	8	53.3
		All seasons	37	33	70	17	45.9	17	51.5	34	48.6
	Young	Middle	14	17	31	3	21.4	1	5.9	4	12.9
		Late	41	16	57	21	51.2	5	31.3	26	45.6
		All seasons	55	33	88	24	43.6	6	18.2	30	34.1
		Mainland overall	92	66		41	44.6	23	34.8	64	40.5
		Overall	133	108		47	35.3	25	23.1	72	29.9

[a]Adults: after hatch-year birds, young: hatch-year birds.

Table 2. Eastern Equine Encephalitis Virus Antibody Seroprevalence Passerine Species Captured at Maine Banding Stations, 2013–2014

				Tested		*Positive*	
Site	*Age*[a]	*Species*	*Season*	*2013*	*2014*	*2013*	*2014*
Island Stopover	Adults	Common Yellowthroat	Early	16	19	2	0
		Gray Catbird	Early	14	8	4	2
		Veery	Early		1		0
		White-throated Sparrow	Early	11	14	0	0
				25	42	6	2
Mainland	Adults	American Robin	Early	1		0	
		American Robin	Middle	2	2	1	0
		American Robin	Late	5	3	2	2
		Gray Catbird	Early	3		2	
		Gray Catbird	Middle	6	9	2	7
		Hermit Thrush	Late	1		1	
		Northern Cardinal	Early	1		0	
		Northern Cardinal	Late	1	1	1	0
		Northern Waterthrush	Early	1		0	
		Northern Waterthrush	Middle	4	3	1	0
		Northern Waterthrush	Late	1		1	
		Song Sparrow	Early	1		0	
		Song Sparrow	Middle	3	5	2	1
		Song Sparrow	Late	3		1	
		Veery	Early	3	4	2	2
		Veery	Middle	1	6	1	5
				37	33	17	17
	Young	American Robin	Middle	1	2	0	1
		American Robin	Late	13	5	3	1
		Gray Catbird	Middle		1		0
		Gray Catbird	Late	10		7	
		Hermit Thrush	Late	7	1	4	0
		Northern Cardinal	Middle		1		0
		Northern Cardinal	Late	5	6	3	2
		Northern Waterthrush	Middle	2	4	0	0
		Ovenbird	Middle		1		0
		Ovenbird	Late		1		1
		Song Sparrow	Middle	1		1	
		Song Sparrow	Late	2		1	
		Veery	Middle	10	8	2	0
		Veery	Late	2	2	2	0
		White-throated Sparrow	Late	2	1	1	1
				55	33	24	6

[a]Adults: after hatch-year birds, young: hatch-year birds.

absence of EEEv activity on the offshore island and/or reversion of migrants during transit. The early season adults sampled from the mainland probably included winter, summer, and year-round residents. Thus while adult birds may somewhat reflect recent interaction with EEEv, young, locally hatched birds should be better sentinels of recent, locally acquired transmission.

Avian EEEv seroprevalence in this study (23–35%) was within the ranges reported by earlier studies in other states (average 23.7%, range 5.5–44.8%; Table 3). Young bird EEEv seroprevalence in this study (34%) was consistent with that of young birds during a period of active EEEv transmission in New Jersey (28%, Crans et al. 1994) and much higher than during an inactive period in New York (1%, Emord and Morris 1984, Table 3). It is possible that EEEv seroprevalence in young birds reflected two consecutive years of high *C. melanura* EEEv positivity in Maine in 2013 and 2014. Samples from young birds in both active and inactive EEEv years are needed to corroborate the relationship between EEEv transmission and avian EEEv antibody levels in young birds.

An effective surveillance program will require consistent, cost-effective, long-term sampling of target bird species. In this pilot study, avian species composition and sample size differed among comparison groups, which introduced unknown bias. Future studies should balance species composition and sample sizes among comparison groups to the extent possible, and incorporate prospective power analyses to guide sample size.

The commonly seropositive species across studies listed in Table 3 were: Gray Catbird, American Robin, Blue Jay, Northern Cardinal, Wood Thrush (*Hylocichla mustelina*), and Tufted Titmouse (*Baeolophus bicolor*). Based on feeding preferences of *C. melanura* in Connecticut, Molaei et al. (2016) found that certain species play key roles as EEEv

Table 3. Eastern Equine Encephalitis Virus Antibody Seroprevalence in Passerines in Various States

			All ages			*Young (hatch year)*		
State	*Study*	*Year*	*Positive*	*Total*	*%*	*Positive*	*Total*	*%*
Maine	This study	2013	47	133	35.3	24	55	43.6
		2014	25	108	23.1	6	33	18.2
		Totals	72	241	29.9	30	88	34.1
Massachusetts	Main et al. (1998)	1959–1970	182	1208	15.1			
New Jersey	Crans et al. (1994)	1990	59	579	10.2			
		1991	17	621	27.7			
		1992	233	520	44.8			
		1993	30	128	23.4			
		Totals	494	1848	26.7	63	226	27.9
New York	Emord and Morris (1984)	1978	182	821	22.2	3	242	1.2
		1979	104	2025	5.1	8	915	0.9
		1980	28	793	3.5	2	272	0.7
		Totals	314	3639	8.6	13	1429	0.9
Maryland	Dalrymple et al. (1972)	1969	601	2866	21.0			
Michigan	McLean et al. (1985)	1980	120	401	29.9			
Alabama	Stamm (1963)	1959–1960	230	631	36.5			

''superspreaders'': American Robin, Tufted Titmouse, Common Grackle (*Quiscalus quiscula*), Chipping Sparrow (*Spizella passerine*), Black-capped Chickadee (*Poecile atricapillus*), Northern Cardinal, Warbling Vireo (*Vireo gilvus*), and especially, Wood Thrush.

Tested birds should include species that seroconvert both rapidly and slowly, and species that breed close to and farther from pathogen foci. High reversion rate species might be good sentinels of EEEv amplification within a season, whereas those with low reversion rates might help track long-term trends. Main et al. (1988) found that the Black-capped Chickadee and Veery demonstrated rapid seroconversion and reversion, whereas antibody was long lasting in the Gray Catbird and Swamp Sparrow. Upland passerine species, living away from *C. melanura* forested wetland habitat, might have higher EEEv seroprevalence in active EEEv years and serve as geospatial sentinels, for example, Field Sparrow (*Spizella pusilla*). Northern Waterthrushes, captured in this study, represent a species likely to live in or near *C. melanura* habitat. We suggest that young, locally hatched ''superspreaders'' mentioned above, plus Gray Catbird and Veery be sampled in future studies. It would be ideal to also sample hardy birds overwintering near *C. melanura* habitat to better understand their role in overwintering of EEEv.

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Author Disclosure Statement

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