Multiple Isolations of Eastern Equine Encephalitis and Highlands J Viruses from Mosquitoes (Diptera: Culicidae) During a 1996 Epizootic in Southeastern Connecticut

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ABSTRACT Thirty-six isolations of eastern equine encephalitis virus were obtained from 8 species of mosquitoes collected from 5 September through 18 October 1996 during an epizootic in southeastern Connecticut. These included *Culiseta melanura* (Coquillett) (19 isolates), *Culex pipiens* L. (8), *Culiseta morsitans* (Theobald) (3), *Aedes sollicitans* (Walker) (2), *Aedes cantator* (Coquillett) (1), *Aedes trivittatus* (Coquillett) (1), *Aedes vexans* (Meigen) (1), and *Coquillettidia perturbans* (Walker) (1). Isolations from *Ae. cantator* and *Ae. trivittaus* are new to North American records, and those from *Ae. cantator* and *Ae. sollicitans* represent the first infections of human-biting, salt-marsh mosquitoes with eastern equine encephalitis virus in Connecticut. With one exception, eastern equine encephalitis-infected *Cs. melanura* were found at all sites where eastern equine encephalitis virus was isolated. The large number of eastern equine encephalitis isolations from *Cs. melanura* and the collection of infected mosquitoes in residential woodlots and coastal salt marshes away from traditional red maple or white cedar swamp habitats, reaffirm the importance of local populations of this mosquito for viral amplification and dispersal from swamp foci. Highlands J virus was more widespread geographically, but fewer isolations of this virus were made from fewer species of mosquitoes. These included *Cs. melanura* (8 isolates), *Cx. pipiens* (5), *Ae. vexans* (3), *Aedes canadensis* (Theobald) (1), *Ae. cantator* (1) and *Cs. morsitans* (1). No human or horse cases of eastern equine encephalitis were reported, although this represents the largest number of isolations for eastern equine encephalitis ever recovered from field-collected mosquitoes in Connecticut.

KEY WORDS eastern equine encephalitis, Highlands J, arbovirus, isolation, mosquito, epizootic

EPIZOOTICS OF EASTERN equine encephalitis have occurred irregularly in Connecticut since 1938 (Andreadis 1993). The majority of these outbreaks have been reported from the eastern portion of the state during late summer and early fall (August–October). Cases of eastern equine encephalitis have been limited to horses and domestic pheasants, and most sites where eastern equine encephalitis have typically been in close proximity to freshwater swamp habitats (red maple or white cedar) or in swamp–forest border locations. There has been no discernible periodicity, but eastern equine encephalitis activity in Connecticut historically has coincided with major outbreaks in humans and horses in southeastern Massachusetts (Edman et al. 1993) and Rhode Island (Gettman 1993). The most recent epizootic in horses (5 cases) occurred in 1990–91 and was confined largely to southeastern Connecticut (Andreadis et al. 1994).

Despite this activity, there have been very few isolations of eastern equine encephalitis virus from field-collected mosquitoes in Connecticut. Wallis et al. (1960) obtained a single isolation from *Aedes vexans* (Meigen) among 11,625 mosquitoes trapped between 1955–59. Main et al. (1979) subsequently made 4 isolations from *Culiseta melanura* (Coquillett) from 157,556 mosquitoes that were collected in areas with a history of eastern equine encephalitis activity in domestic animals over a 10-yr period, 1969–1978. These isolations were coincident with the reporting of multiple human and horse cases in southeastern Massachusetts during 1970–1975 (Grady et al. 1978). More recently, Andreadis et al. (1994) obtained no isolations of eastern equine encephalitis from 21,347 mosquitoes (21 species in 7 genera) collected from areas in southeastern Connecticut where confirmed horse cases had occurred the previous year.

In late August and early September 1996, an unusually large number of eastern equine encephalitis-infected mosquitoes was detected in and around a presumed focus at Chapman Swamp in Westerly, Rhode Island (Markowski 1996). In response to this finding, we initiated an emergency mosquito arbovirus surveillance program in adjacent regions of southeastern Connecticut. Our objectives were to determine the identity, distribution, and relative abundance of eastern equine encephalitis-infected mosquitoes in this region. The results of this investigation are reported next.

Materials and Methods

Mosquito Collection and Identification Techniques. Mosquito trapping was conducted at 80 different locations in 20 townships (Fig. 1) from 5 Sep-

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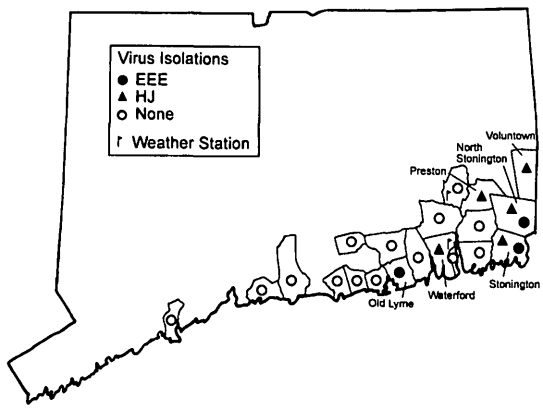

Fig. 1. Connecticut state map showing towns where mosquitoes were collected for arbovirus testing and those sites where EEE and Highland J (HJ) viruses were isolated.

tember through 18 October 1996. The majority of these sites were in the southeastern portion of the state and included mixed hardwood swamps, isolated woodlots in residential areas, and coastal salt marshes.

Adult female mosquitoes were collected with dry ice-baited CDC miniature light traps. One trap per site per night was used, and the number of trap nights per site ranged from 1 to 10 (ave. = 2), with the most repetitive sampling generally occurring at those locations where eastern equine encephalitis virus isolations were made. Mosquitoes were transported live to the laboratory where they were immediately frozen on dry ice and then identified on a chill table using the keys of Carpenter and LaCasse (1955), Darsie and Ward (1981) and Means (1979, 1987). Specimens were pooled by species, site, and collection date. The number of mosquitoes per pool was ≤50 and minimum field infection rates (number of positive pools per total number of mosquitoes tested) were calculated for each species at specific locations.

Rainfall and temperature data were obtained from 2 National Weather Service Climatological Stations (Groton and Norwich Public Utilities Plant) that were located close to the collection sites (Fig. 1).

Virus Assays. Each frozen mosquito pool was homogenized in phosphate buffered saline containing 0.5% gelatin, 30% rabbit serum, antibiotic, and antimycotic. The homogenate was centrifuged for 10 min at 520 g to clear the mixture of mosquito debris. A 0.1-ml aliquot of each supernatant then was inoculated into a 25-cm^2 flask containing a monolayer of Vero cells and incubated at 37°C in 5% CO_2 for up to 7 d (Tesh et al. 1992). One uninoculated flask was kept as a negative control. The remainder of the supernatant was stored at −70°C.

Flasks were examined daily for cytopathic effect. If cytopathic effect was noted, the cells were scraped from the flask and a cell lysate antigen was prepared (Ansari et al. 1993). Isolates were identified by enzyme immunoassy using reference antibodies that were prepared in mice and provided by the World Health Organization Center for Arbovirus Research and Reference, Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, Yale University School of Medicine. These included: Cache Valley, eastern equine encephalitis, Highlands J, Jamestown Canyon, La Crosse, and St. Louis encephalitis virus antibodies. Positive control cell lysates were run at least daily. Highlands J and eastern equine encephalitis antibodies crossreact in the enzyme immunoassy, but were distinguishable on the basis of titer and speed of color development. Identity of selected Highlands J and eastern equine encephalitis isolates was confirmed by plaque reduction neutralization test in Vero cells.

Results

The mosquito collection and virus isolation data are shown in Table 1 and the specific locations of the collection sites are detailed in Figs. 1 and 2. In total, 6,440 female mosquitoes representing 16 species in 7 genera were collected from the field and assayed for arboviruses. The most abundant mosquito species were *Cs. melanura* and *Culex pipiens* L. Eight species of *Aedes* were also collected, among which, 2 saltmarsh species, *Aedes cantator* (Coquillett) and *Aedes sollicitans* (Walker) and 2 freshwater species, *Ae. vexans* and *Aedes canadensis* (Theobald), were the most common. *Uranotaenia sapphirina* (Osten Sacken) also was relatively abundant. Comparatively few *Culiseta morsitans* (Theobald) and *Coquillettidia perturbans* (Walker) were trapped.

Thirty-six isolations of eastern equine encephalitis (from 8 mosquito species) and 19 isolations of Highlands J (from 6 mosquito species) were made. There were no isolations of Cache Valley, Jamestown Canyon, La Crosse, or St. Louis encephalitis. With the exception of a single isolation from *Ae. sollicitans* at a salt marsh on Great Island in Old Lyme, all of the eastern equine encephalitis isolations were made from mosquitoes collected in the towns of N. Stonington and Stonington. Highlands J was more widespread geographically, occurring in 5 different townships.

The lst isolations of eastern equine encephalitis were made on 8 September from *Cs. melanura* and *Cx. pipiens*. Virus isolations from these 2 species were made repeatedly thereafter through 30 September (*Cs. melanura* = 19, *Cx. pipiens* = 8). Eastern equine encephalitis isolations from *Cq. perturbans* and saltmarsh *Ae. cantator* and *Ae. sollicitans* were made in mid-September (12–16th), whereas isolations from flood water *Aedes trivittatus* (Coquillett) and *Ae. vexans* were made 2 wks later (28 September).

Site-specific MFIRs ranged from 1:1 to 1:204 and were generally quite high with 4 species, *Cq. perturbans* (1:1), *Cs. melanura* (1:2), *Cs. morsitans* (1:7), and *Cx. pipiens* (1: 7), exceeding 1: 10 in some locations. Eastern equine encephalitis-infected mosquitoes were found at 6 of 13 collection sites in Stonington, mostly in the Pawcatuck and Barn Island areas, and at 3 of 8 collection sites in North Stonington, mostly along the Rhode Island border. These sites were scat-

Table 1. Eastern equine encephalitis (EEE) and Highlands J (HJ) virus isolation data from field-collected female mosquitoes trapped in southeastern Connecticut from 5 September–18 October 1996

Species	No. tested		EEE				HJ			
	Specimens	Pools	No.	Date	Location	MFIR[a]	No.	Date	Location	MFIR
Ae. canadensis	270	22	—				1	9–9	Stonington-10	1:1
Ae. cantator	316	43	1	9–16	Stonington-2	1:48	1	9–12	Stonington-2	1:48
Ae. cinereus	63	11	—				—			
Ae. sollicitans	307	31	1	9–12	Stonington-2	1:74	—			
			1	9–13	Old Lyme	1:34				
Ae. taeniorhychus	152	17	—				—			
Ae. triseriatus	126	31	—							
Ae. trivittatus	30	7	1	9–28	N. Stonington-8	1:22	—			
Ae. vexans	475	74	1	9–28	N. Stonington-8	1:25	1	9–17	Stonington-4	1:28
							1	9–17	Stonington-5	1:34
							1	10–10	Stonington-2	1:67
An. punctipennis	94	46	—				—			
An. quadrimaculatus	4	4	—				—			
Cq. perturbans	46	14	1	9–12	Stonington-2	1:1	—			
Cx. pipiens	1,876	138	1	9–8	Stonington-5	1:74	2	9–17	Stonington-5	1:37
			1	9–9	N. Stonington-7	1:7	1	9–22	Waterford	1:71
			3	9–10, 12, 16	Stonington-2	1:67	1	10–3	Preston	1:10
			1	9–16	Stonington-3	1:72				
			1	9–17	Stonington-4	1:105				
			1	9–17	Stonington-6	1:52				
Cs. melanura	2,322	112	1	9–8	Stonington-1	1:76	1	9–6	Voluntown	1:99
			1	9–9	N. Stonington-7	1:68	2	9–9, 16	N. Stonington-8	1:71
			7	9–10, 12, 16	Stonington-2	1:55	2	9–10, 17	Stonington-4	1:182
			2	9–12, 28	N. Stonington-8	1:77	1	9–16	N. Stonington-7	1:68
			2	9–16, 17	Stonington-3	1:137	1	9–16	Stonington-2	1:386
			3	9–17	Stonington-4	1:122	1	9–17	Stonington-5	1:204
			1	9–17	Stonington-5	1:204	1	10–3	Preston	1:10
			1	9–17	Stonington-6	1:107				
			1	9–30	N. Stonington-9	1:2				
Cs. morsitans	36	9	2	9–10, 16	Stonington-2	1:7	1	9–17	Stonington-4	1:2
			1	9–16	Stonington-3	1:13				
Ps. ferox	1	1	—				—			
Ur. sapphirina	322	60	—				—			
Totals	6,440	620	36				19			

MFIR, Minimum field infection rate. —, No virus isolations

tered within a 17-km radius of one another and, with one exception, were within 6.5 km of Chapman Swamp in Westerly, Rhode Island. The most remote site where eastern equine encephalitis-infected mosquitoes were found in this area was near Wyassup Lake in the Pachaug State Forest (site 9). This site was 13 km northwest of Chapman Swamp. The Old Lyme site by comparison, was ≈40 km west of Barn Island.

The largest number of eastern equine encephalitis isolations were made at Barn Island (sites 1 and 2), a coastal wildlife conservation area in the southeastern most corner of the state. In total, 16 isolations were obtained from 6 of 7 mosquito species that were collected at this location. Twelve isolations also were made from *Cs. melanura* (7), *Cx. pipiens* (4), and *Cs. morsitans* (1) collected in several isolated woodlots adjacent to schools and residential neighborhoods in Stonington (sites 3–6). Three quarters (27 of 36) of the total eastern equine encephalitis isolations were obtained from *Cs. melanura* and *Cx. pipiens*, and eastern equine encephalitis-infected *Cs. melanura* were found at all but 1 site (Old Lyme) site where eastern equine encephalitis was detected.

As with eastern equine encephalitis, the majority of the Highlands J isolations (68%) were made from *Cs. melanura* and *Cx. pipiens*. *Aedes canadensis* (Theobald) was the only species found positive for Highlands J that was not also positive for eastern equine encephalitis. Minimum field infection rates were highly variable depending on the species and the collection site ranging from 1:1 for *Ae. canadensis* (Stonington-10) and *Cx. pipiens* (Preston) to 1:386 for *Cs. melanura* (Stonington-2).

Culiseta melanura was abundant throughout September and no declines in trap catches were seen until the end of the month. Minimum field infection rates of eastern equine encephalitis for this mosquito at sites in Stonington where consecutive collections were made for the entire 6-wk period (from 8 September to 18 October), steadily increased throughout the month ranging from 1:255 on 8 September to 1:41 on 28 September. Substantially fewer adults were collected in October ($n = 79$ versus $n = 1{,}412$) and no isolations were made. No clear trends were discernible among the other species. However, the highest minimum field infection rates for eastern equine encephalitis were detected in mid-September (1:17), and where comparatively large trap catches (mostly *Ae. vexans* and *Cx. pipiens*) were made in October, no eastern equine encephalitis isolations were made after 28 Sep-

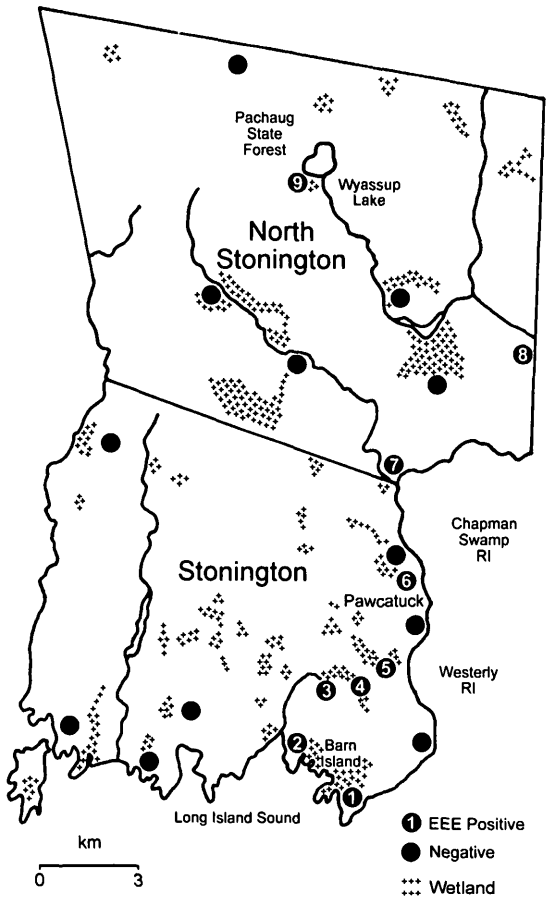

Fig. 2. Map of Stonington and North Stonington showing specific locations where mosquito collections were made and where EEE was isolated.

tember. By contrast, 2 isolations of Highlands J were made from *Cs. melanura* and *Cx. pipiens* on 3 October.

An analysis of precipitation data for the region showed substantially higher than normal rainfall amounts during the months of April (total = 18.3 cm, +7.2 cm), July (total = 15.0 cm, +6.2 cm), and September (total = 17.9 cm, +8.5 cm). Nearly half of the rainfall in July (6.4 cm) was associated with a coastal hurricane (Bertha) that affected the region on 13 July. The rainfall for August was below average (total = 6.5 cm, −3.2 cm). The average daily temperatures recorded in September ranged from a high of 22.4°C to a low of 13.2°C with an overall average of 17.8°C.

Discussion

The 36 isolations of eastern equine encephalitis obtained in our investigation from 8 different species of mosquitoes in southeastern Connecticut were unprecedented. Although arbovirus surveillance has not been conducted regularly in this region, it is significant that only 5 previous isolations of eastern equine encephalitis had been made from nearly 200,000 female mosquitoes that had been collected from areas with a history of eastern equine encephalitis activity in horses and pheasants over a 37-yr period, from 1955 to 1992 (Wallis et al. 1958a, b, 1960; Main et al. 1979; Andreadis et al. 1994). Our isolations from *Ae. sollicitans, Cs. morsitans, Cx. pipiens,* and *Cq. perturbans* represent new state records and, to our knowledge, those from *Ae. cantator* and *Ae. trivittatus* are new North American records.

The large number of eastern equine encephalitis isolations from *Cs. melanura* and the collection of infected mosquitoes in residential woodlots and in areas bordering coastal salt marshes away from traditional red maple or white cedar swamp habitats, reaffirm the importance of local populations of *Cs. melanura* for viral amplification and dispersal from swamp foci (Morris et al. 1980; Howard et al. 1988, 1989, 1996). Howard et al. (1996) showed that in central New York, eastern equine encephalitis-infected *Cs. melanura* disperse from swamp foci to upland areas and function as a mechanism for introducing virus into adjacent swamp complexes. Once virus is introduced into an area, local populations of *Cs. melanura* may amplify the virus in resident bird populations which then serve as a virus source for other mosquitoes (*Aedes* spp. *Cq. perturbans*) that can transmit virus to horses or humans. Given an effective fight range of 4–9 km for *Cs. melanura* (Howard et al. 1989), the discontinuous distribution pattern of our isolations also supports the supposition for multiple foci of eastern equine encephalitis in this region of southeastern Connecticut as has been documented in the Toad Harbor and Cicero Swamp regions of central New York (Howard et al. 1996).

The isolations from *Ae. cantator* and *Ae. sollicitans* collected from salt marsh habitats were particularly noteworthy, because most sites where eastern equine encephalitis historically has occurred in Connecticut, have been in or adjacent to inland fresh-water swamps (red maple or white cedar) (Wallis 1958a; Main et al. 1979; Andreadis 1993). Our findings thus represent the first incrimination of human-biting, salt-marsh mosquitoes in the epizootiology of eastern equine encephalitis in this region. *Aedes sollicitans* has been implicated in the transmission of eastern equine encephalitis to humans in New Jersey (Crans et al. 1986), and it is among the most abundant and pestiferous mosquitoes found in coastal areas of Connecticut. Although *Ae. cantator* has not been incriminated in human or horse transmission of eastern equine encephalitis, this mosquito has been shown to be a competent vector in laboratory tests (Merrill et al. 1934; Davis 1940) and it was suspected as a possible vector in the 1938 epidemic in Massachusetts (Feemster and Getting 194; Getting 1941). These observations argue for the inclusion of coastal salt-marsh habitats in any future surveillance for eastern equine encephalitis activity in the state, especially if they are adjacent to freshwater swamps that support *Cs. melanura* populations.

Unlike *Ae. cantator* and *Ae. sollicitans,* the vector competency of *Ae. trivittatus* for eastern equine en-

cephalitis has not been ascertained in laboratory tests and, prior to our study, no eastern equine encephalitis virus isolations had ever been made from field-collected females of this species. Therefore, although *Ae. trivittatus* is a persistent biter that readily will feed on humans (Carpenter and LaCasse 1955), its role as a potential bridge vector to horses or humans is unknown.

Our 11 eastern equine encephalitis isolations from *Cs. morsitans* (3) and *Cx. pipiens* (6) are consistent with the blood-feeding preference of these species for passeriform birds (Magnarelli 1977), and their late-season prominence at fresh-water swamp sites where eastern equine encephalitis activity historically has occurred (Wallis et al. 1958a; Morris and Zimmerman 1981). Although *Cs. morsitans* is univoltine, it is long-lived and adult populations extend from mid-May through September (Morris et al. 1976; Morris and Zimmerman 1981). According to Morris and Zimmerman (1981), its ornithophagic blood-feeding habits preclude any major role as a vector to non-avian hosts, but its vectoral capacity for amplification of eastern equine encephalitis virus among birds parallels that of *Cs. melanura* and is only diminished by its comparatively lower abundance after mid-August.

Eastern equine encephalitis virus rarely has been isolated from *Cx. pipiens* (Morris et al. 1975; Srihongse et al. 1980) and the significance of this mosquito in the epizootiology of eastern equine encephalitis has not been clearly established. Merrill et al. (1934) and Davis (1940) were unable to obtain laboratory transmission with this species, the latter reporting the disappearance of the virus from blood-fed mosquitoes within 2–3 d. On the other hand, the abundance of this species and large number of isolations found in the present study indicate that it could be a more important epizootic vector than is currently recognized. Although unable to isolate eastern equine encephalitis virus from *Cx. pipiens* during outbreaks that occurred in Connecticut from 1953 to 1956, Wallis et al. (1958a) reported it to be the most abundant species collected in and around afflected pheasant farms in September and early October.

A lower number of Highlands J than eastern equine encephalitis isolations was obtained during our investigation. This virus produces an apparently benign infection in humans and horses (Chamberlain 1980), but has the same fundamental epizootiology as eastern equine encephalitis (Hayes and Wallis 1977). Highlands J is maintained in an enzootic cycle involving *Cs. melanura* mosquitoes and wild passerine birds that inhabit fresh-water swamps. Peak periods of transmission, as demonstrated by the isolation of virus from mosquitoes, generally occur in the late summer and early fall. Long-term surveillance in Massachusetts swamps has shown that Highlands J is almost always more prevalent than eastern equine encephalitis, and it is usually detected earlier in the season. However, in the current study, Highlands J was more widespread geographically than eastern equine encephalitis, but it was not nearly as prevalent. On the other hand, nearly three-quarters (14 of 19) of the HJ isolations were made in the same locales where eastern equine encephalitis was found. Therefore, although there does appear to be some relationship between the occurrence of these 2 viruses, the usefulness of Highlands J virus isolations as a reliable predictor of impending eastern equine encephalitis activity is, at best, uncertain.

Despite the large number of eastern equine encephalitis virus isolations that were obtained in our investigation, no human or horse cases were reported in the region. However, confirmed deaths were recorded for 2 emus, *Dromaius novaehollandiae* Linn. at a farm in Hopkinton, Rhode Island (Markowski 1996). This farm was <3 km from a collection site in North Stonington (no. 8) where we recovered eastern equine encephalitis from *Ae. trivittatus*, *Ae. vexans*, and *Cs. melanura*.

In the absence of a concurrent serologic survey, it is difficult for us comment on the apparent absence of human cases. Given the extraordinary number of virus isolations that were recovered from human-biting mosquitoes in many populated areas, we would have anticipated some human involvement. Inapparent human infections with eastern equine encephalitis have been noted in New Jersey (Goldfield et al. 1968a), Louisiana (Schaeffer et al. 1954) and Massachusetts (Feemster et al. 1958), but inapparent infection rates are usually low (from 0.5 to 7.3%). However, Goldfield et al. (1968b) computed a ratio of 16–32 inapparent infections for every overt case during the 1959 eastern equine encephalitis outbreak in New Jersey, with an attack rate in humans of ≈1/1,000. The absence of inapparent human infections in this region of southeastern Connecticut needs to be determined.

Because we have no data on the presence of virus in reservoir bird populations and no mosquito or virus isolation data from July, August, or from prior years, it is equally difficult for us to speculate on what led to the apparent amplification and concentration of eastern equine encephalitis in mosquitoes in this region. Higher than normal rainfall amounts were recorded in April and July, but August was drier than normal. However, the region did experience an early coastal hurricane (Bertha) on 13 July. Whether the associated winds played a role by possibly introducing eastern equine encephalitis-infected birds or mosquitoes is unknown, but it is one hypothesis. However, this region has a history of horse and pheasant cases dating back to 1938 (also in 1947, 1951, 1953, 1956, 1959, and 1972) (Andreadis 1993), and although no eastern equine encephalitis activity before 1996 had been recorded for 24 yr, this region does appear to be an occasional focus for virus activity. Continued surveillance of mosquito populations in this region of southeastern Connecticut is clearly warranted.

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