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ABSENCE OF EASTERN EQUINE ENCEPHALITIS (EEE) VIRUS IN IMMATURE *COQUILLETTIDIA PERTURBANS* ASSOCIATED WITH EQUINE CASES OF EEE[1]

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During the summer of 1983, 5 fatal equine cases of eastern equine encephalitis (EEE) were confirmed in New Jersey (Crans, unpublished data). These cases were conspicuous by their occurrence at sites, ca. 30 km from the nearest salt marsh, in Ocean and Sussex counties. Historically, equine cases of EEE have been repeatedly diagnosed from several inland localities in New Jersey (Goldfield and Sussman 1968). Hayes et al. (1962) indicated that the distance from coastal marshes made it unlikely that the salt marsh mosquito, *Aedes sollicitans* (Walker), served as the vector for EEE virus in these areas.

Four of the 1983 cases occurred at a distinct focus in Jackson Township, Ocean County. After the initial case with an onset of July 8, mosquito surveillance was initiated and concentrated on 3 Jackson Township farms within a 5-km radius of each other. Subsequent cases from this area had onsets of July 23, August 12 and August 26. The dominant mosquito species collected at these farms was *Coquillettidia perturbans* (Walker), ranging from 77% of the total in July to 44% in September. From the 2,158 mosquitoes assayed from this area by the New Jersey State Department of Health, 2 isolates of EEE virus were obtained from 1,225 *Cq. perturbans*. These viral isolates, from collections made on July 25 and September 7, reflected a minimum field infection rate (MFIR) in this area of 1:612 for this species. Three additional EEE virus isolates were obtained from 263 *Culiseta melanura* (Coquillett) (MFIR = 1:88).

The first reported isolation of EEE virus from an arthropod was from *Cq. perturbans* collected in Georgia in 1948 (Howitt et al. 1949) and EEE virus has since been isolated from this species in New York (Morris and Srihongse 1978) and New Jersey (Veazey et al. 1980). *Coquillettidia perturbans* was likely responsible for transmission of EEE virus to equines and humans in southwestern Michigan in 1980 (Francy 1982) and has a high vector potential for this virus in Massachusetts (Nasci and Edman 1981). Field studies of the blood-feeding pattern for this species have linked it closely to large mammals, especially equines (Nasci and Edman 1981).

In laboratory studies with *Cq. perturbans* and EEE virus, Chamberlain et al. (1954) demonstrated virus transmission by this species and gave it a "good vector potential rating." Schaeffer and Arnold (1954) found that EEE virus, ". . . could be demonstrated in eggs laid by infected (*Mansonia* [*Coquillettidia*] *perturbans*." Following oral infection of *Cq. perturbans* with Venezuelan equine encephalitis (VEE) virus, Chamberlain et al. (1956) recovered VEE virus from 7 of 16 egg rafts. In a similar study, they found that 2 of 19 egg rafts laid by *Cq. perturbans*, after ingesting an infectious blood meal, contained EEE virus. In an attempt to preclude transovum viral contamination, Chamberlain and coworkers rinsed the surface of the eggs they assayed with a serum-saline solution. The difficulty associated with laboratory colonization of *Cq. perturbans* has prevented any definitive studies of its role as an overwintering host for arboviruses.

The association of EEE virus with equine fatalities in New Jersey during 1983 suggested a more important role for *Cq. perturbans* in the ecology and transmission of this virus. The maintenance of the virus by larval mosquitoes has been proposed as an overwintering mechanism during interepidemic periods. Therefore, we viewed this as an opportunity to explore the hypothesis that EEE virus was maintained through the winter in *Cq. perturbans* larvae.

From October 1983 through March 1984, immature *Cq. perturbans* were collected in areas adjacent to the sites where the 4 Ocean County

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[1] The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense. New Jersey Agric. Expt. Sta. Publ. No. D40101-01-85, supported by funds from the New Jersey State Mosquito Control Commission and the Hatch Act.

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equine fatalities occurred. Additional collections were made in Cape May and Gloucester counties where EEE virus transmission has been detected intermittently during prior years. Larvae were removed from submerged roots of a variety of aquatic shrubs, herbaceous plants, and grasses by utilizing a modified bilge pump according to the methods of Walker and Crans (personal communication). Larvae were separated with soil survey sieves and pooled by stage in 1 ml of diluent containing medium H-199 supplemented with glutamine, 20% fetal bovine serum (FBS), and antibiotics (200 units/ml penicillin, 200 ug/ml streptomycin, and 10 μg/ml fungizone). In order to avoid a possible inhibitory effect on the virus by the larvae, as reported by Ksiazek et al. (1985) for several virus-vector systems and that was subsequently verified for *Cq. perturbans* and EEE virus (Clark et al. unpublished data), 2nd stage larvae were placed in pools of 20 while 3rd and 4th stage were limited to 10 per pool. All pools were sealed and stored on dry ice or at −70°C until assayed for virus.

Each pool of *Cq. perturbans* was ground in a prechilled tissue grinder. Suspensions were clarified by centrifugation at 2,500 rpm for 20 min at 4°C, and the resultant supernatants were assayed for virus by plaque assay in Vero cells. Aliquots of 0.1 ml of each supernatant were inoculated in triplicate into Vero 12-well trays and adsorbed for 1 hr at 35°C and then overlaid with 2 ml of agar overlay media consisting of equal volumes of 1.5% agarose and 2x Eagle's basal medium in Earle's salts with Hepes buffer (4 g/liter), 10% heat-inactivated FBS, and PSF. Cell cultures were incubated in a humidified atmosphere with 5% CO_2 for plaque development. After 4 days, 1 ml of the overlay medium containing a 7% solution (stock = 1:300) of neutral red was added to each cell monolayer. These cultures were incubated for 24 hr and examined for plaques.

Virus was not isolated from 6,560 immature *Cq. perturbans* (Table 1). The majority (71%) of these larvae originated from Ocean County near sites where the 4 equine fatalities had occurred.

Although EEE virus surveillance was initiated on June 1, 1984, evidence of EEE virus transmission at the Jackson Township site was not detected until August 24. Virus transmission continued through September 28, based on 7 EEE virus isolations from *Cs. melanura* during this period. At the Dennisville site in Cape May County, EEE virus activity was indicated by recovery of 25 isolations from *Cs. melanura* between August 2 and September 27. Additionally, human EEE cases were confirmed during 1984 in individuals exposed approximately 30 km northeast and northwest of this study site. In contrast to the previous year, adult populations of *Cq. perturbans* were greatly reduced in 1984, despite large larval collections at these two localities. In 1984, 5 fatal equine cases of EEE were reported from Gloucester County where only 200 immature *Cq. perturbans* were collected during November 1983. In general, there was substantial EEE virus activity detected in these areas during the summer following this study of immature *Cq. perturbans.*

Table 1. Immature *Coquillettidia perturbans* collected in New Jersey and assayed for virus.

Collection areas	Collection period	Stage	No. assayed	No. pools
Dennisville, Cape May Co.	Nov. 1983–Jan. 1984	L2	922	47
		L3	701	70
		L4	70	7
		Pupa	2	1
	Subtotal		1,695	125
Iona Lake, Gloucester Co.	Nov. 1983	L2	120	6
		L3	70	7
		L4	10	1
	Subtotal		200	14
Jackson and Mills Lake, Ocean Co.	Oct. 1983–Mar. 1984	L2	1,415	111
		L3	3,000	300
		L4	250	25
	Subtotal		4,665	436
	TOTAL		6,560	575

This study of the overwintering stages of *Cq. perturbans,* temporally and spatially associated with equine fatalities of EEE and EEE virus-infected female *Cq. perturbans,* failed to implicate this species as an overwintering host for EEE virus. With Keystone virus (LeDuc et al. 1975), which is transovarially transmitted, the MFIR in reared adults approximates that found in larvae. If transovarial transmission was occurring in the virus-vector system we investigated and the 2 EEE virus isolates (MFIR = 1:612) were the result of transovarial transmission infections, we should have obtained 7 isolates from the Jackson Township collections. Although *Cq. perturbans* appears to play a major role as an epizootic vector for EEE virus in freshwater habitats, we were not able to detect transovarial or transstadial maintenance of EEE virus in this species.

We thank Drs. Michael D. Turell and Douglas M. Watts for reviewing the manuscript.

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