ARTICLES

STUDY OF HIBERNATING MOSQUITOES IN EASTERN EQUINE ENCEPHALOMYELITIS EPIDEMIC AREAS IN CONNECTICUT

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One of the most interesting problems in the natural history of the arthropod-borne viruses is their mechanism of survival during inter-epidemic periods. This problem applies to eastern equine encephalomyelitis (EEE) in the New England States where virus has not been isolated except during the epidemic season. In 1956, Blackmore and Winn (1) reported isolation of western equine encephalomyelitis (WEE) virus from one of 31 pools of *Culex tarsalis* collected during the winter in Colorado and suggested that under certain conditions WEE virus might persist from one active transmission season to the next within overwintering mosquitoes.

Study of the possibility of the EEE virus overwintering in New England in hibernating mosquitoes was begun in 1955, when 640 hibernating *Culex pipiens* were examined for virus with negative results (2). The purpose of this report is to present results of further study of overwintering mosquitoes collected in the proximity of farms in Connecticut where there has been confirmed virus activity.

PROCEDURE. Mosquito hibernation places were found on or near seven farms which had a history of EEE virus activity. Details of these study sites are given in Table 1. Collection trips were made to the areas as long as hibernating mosquitoes were found during the period from September 1, 1956, to April 1, 1957. An average of two areas per week were visited, depending upon weather conditions. Care was taken to omit specimens of nonhibernating species which were occasionally found taking shelter in the hibernation sites during the early portion of the winter season. Only specimens which appeared to contain fat bodies and were believed to be hibernating were included in this study. Adult female mosquitoes were collected by hand aspirator and transported in cardboard cartons to the laboratory, where they were maintained alive at room temperature (76±4° F.) for three to five days. During this period they were provided with cotton pads saturated with 5 percent sucrose solution for food. At the end of this time the mosquitoes were stupefied with cigarette smoke, sorted into pools by species (Table 2), placed in small vials and stored in refrigeration (4° C.) overnight until transported to the virus laboratory for virus isolation.

Virus Isolation Procedures. The mosquito pools were triturated either in a porcelain mortar with pestle or in a Ten Broeck grinder, and suspended in the proportion of one mosquito to 0.2 ml. of Hank's balanced salt solution which contained antibiotics and was enriched by the addition of 0.5 percent lactalbumin hydrolysate and 2 percent calf serum (3). The suspension was centrifuged at 10,000 rpm for 20 minutes and the supernatant used for inoculation.

All of the 73 mosquito pools were inoculated into tissue culture and 31 were also inoculated intracerebrally (i.c.) into litters of one to three day old mice.

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TABLE 1.—Collection sites in Connecticut from which hibernating mosquitoes were collected during 1956–1957

Collection Site No.	Place	Location in State	Hibernation Site	History of EEE Activity * in Proximity of Collection Site
1	Barn Island, Stonington	East-Coastal	Stone basement under abandoned house	Pheasant deaths—1947, 1951 Horse deaths —1938, 1953, 1956
2	Ked's Cave, Branford	Central-Coastal	Deep natural cave	Pheasant deaths—1951
3	Sherwood Island, Fairfield	West-Coastal	Stone outbuildings in State Park	Pheasant deaths—1956
4	Roxbury Iron Mine, Roxbury Falls	West-Central	Abandoned mine shaft	Horse death —1938
5	Shade Swamp Wildlife Management area, Farmington	Mid-Central	Eight artificial stone caves (shallow)	Pheasant deaths—1953 Rodent death —1953
6	Fitch's Farm, Jewett City	East-Central	Old stone basement under a house	Pheasant deaths—1955–1956 Horse death —1955
7	State Prison Farm, Enfield	North-Central	Old stone basement under fertilizer barn	Pheasant deaths—1956

* Confirmed by isolation of EEE virus.

Tissue cultures were prepared by mincing (with scissors) ten-day-old chick embryos (head removed) and washing them twice in buffered saline to remove excess red blood cells by allowing tissue fragments to settle, and discarding supernatant. The tissue was then treated with 0.2 percent trypsin in buffered saline at 37° C. for 20 minutes in a magnetic stirrer and filtered through two layers of gauze to remove the large fragments of the embryonic tissue. The suspended cells in the filtrate were sedimented by centrifugation for eight minutes at 800 rpm and washed twice with buffered saline solution; and finally, 1 ml. of packed cells was suspended in 499 ml. of enriched Hank's solution. The suspended cells were then distributed in 1 ml. amounts in culture tubes and the tubes tightly plugged with rubber stoppers.

The supernatant of the mosquito pools was inoculated in amounts of 0.1 ml. to each of three or more culture tubes. The tubes were incubated in a slanted position at 37° C. and examined every other day for six or more days.

Whether cytopathogenic effect (CPE) was observed or not, a second passage was made from a pool of the initially inoculated tubes and only after the second passage failed to reveal CPE were the results recorded as negative.

An account of experiences with tissue culture in the detection of EEE virus will be published later. It will suffice to say here that in this laboratory the tissue culture method has compared favorably with the infant mouse inoculation method.

RESULTS. From the seven collection sites listed in Table 1, a total of 2,569 hibernating mosquitoes were obtained. The number of pools and number of each species

TABLE 2—Hibernating mosquito species collected in Connecticut, number of pools and number of specimens tested for EEE virus, 1956–1957

Species	No. of Pools	No. of Specimens	Virus Isolation Results
Culex pipiens	33	1337	neg.
Culex salinarius	21	910	neg.
Culex restuans	6	115	neg.
Culex territans	6	33	neg.
Anopheles quadrimaculatus	3	132	neg.
Anopheles punctipennis	4	42	neg.
Total	73	2569	0

are listed in Table 2. From a total of 73 pools, 33 (1,337 specimens) were of *Culex pipiens*—the species encountered in greatest abundance in hibernation sites. A total of 21 pools of *Culex salinarius* (910 specimens) were processed. These were primarily from collections obtained from Site #1 at Barn Island, in southern Connecticut, where this species was particularly abundant. The remaining 18 pools consisted of various numbers of four other species (Table 2) which were found hibernating throughout the winter. From the total of 2,569 specimens studied, no virus isolations were obtained.

DISCUSSION. This study had two objectives relating to the question of EEE virus overwintering in Connecticut in hibernating mosquitoes. The first was to obtain information on the number and species of mosquitoes which hibernate in areas where EEE virus was known to have been active, and the second was to ascertain if these mosquitoes harbored EEE virus.

Notwithstanding the difficulties involved in locating hibernating sites for mosquitoes, it is felt that this study rather clearly indicates that the number of mosquito species which commonly hibernate in the adult stage in Connecticut is quite limited. In fact, only six species (Table 2) were found. Of these, only *Culex restuans* is known to be capable of transmission of the virus (4). While virus isolations have been obtained from *Culex salinarius,* (5) transmission by this species is considered unlikely (4). During the periods of active transmission, the virus has been most frequently isolated from *Culiseta melanura* (5, 6, 7, 8) and this mosquito is presumed to be the most important vector. However, no hibernating specimens of this species were found and there is no other evidence that this mosquito hibernates in the adult stage. Among those suspected of being capable of transmitting the virus only mosquitoes of the genus *Culex* were found hibernating. Barr (9) has pointed out that after a blood meal has been taken it seems improbable that *Culex* mosquitoes can undergo the physiologic adaptation process (gonadotropic-dissociation) which is necessary to convert from egg production to hibernation. Since, so far as is known, it is necessary that a blood meal from an infected host be taken by the mosquito in order for it to become infected, it is highly improbable that the virus can be harbored by hibernating mosquitoes of this genus.

The failure to isolate virus from the rather limited number of mosquitoes examined may not be of significance. Yet it should be remembered that these mosquitoes were collected in places where the environment was favorable to the propagation of the virus during previous years. In addition, due to the vast reduction in the mosquito population during the winter season, small numbers in collections represent a proportionally greater sample of the population than do large collections during the summer season when the population is greatly expanded. Considered alone, this negative finding bears no great weight, but it is in keeping with other evidence of a

negative nature. Although these studies have unearthed no evidence favoring the concept of EEE virus overwintering in hibernating mosquitoes, admittedly they do not eliminate the possibility of this occurring.

SUMMARY. During the winter of 1956–57 hibernating mosquitoes were collected from seven localities in Connecticut where there had been history of eastern equine encephalomyelitis (EEE) virus activity. The 2,569 specimens collected represented six species: *Culex pipiens* 1,337, *Culex salinarius* 910, *Culex restuans* 115, *Culex territans* 33, *Anopheles quadrimaculatus* 132, and *Anopheles punctipennis* 42.

The mosquitoes were currently pooled according to species, triturated and inoculated into chick embryo tissue cultures for detection of virus. Thirty-one of the 73 pools were also inoculated into infant mice. No virus was isolated.

The implication of these observations upon the overwintering of EEE virus in hibernating mosquitoes in Connecticut is discussed.

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COLONIZATION OF *AEDES TAENIORHYNCHUS*

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Salt-marsh mosquitoes, *Aedes taeniorhynchus* (Wied.) and *A. sollicitans* (Walk.), have developed resistance to DDT, BHC, and dieldrin in several coastal areas of Florida where these insecticides have been used extensively. Laboratory investigations of more effective materials and methods of control for these species have been carried out in the past with field-collected larvae and pupae. These investigations have been hampered by the irregular occurrence of broods, even during the normal breeding season, and by difficulty in getting sufficient numbers in suitable condition for testing. For these reasons the laboratory studies could not be carried on continuously in keeping with the importance of the problem.

To meet the demands for specimens, insectary rearing of *taeniorhynchus* was begun at the Orlando, Fla., laboratory in April, 1957. The colony was started with field-collected eggs, larvae, and pupae from salt marshes in Brevard and Volusia Coun-