EXPERIMENTAL EVIDENCE AGAINST THE TRANSOVARIAL TRANSMISSION OF EASTERN EQUINE ENCEPHALITIS VIRUS IN *CULISETA MELANURA*[1]

HENRY E. SPRANCE[2]

University of Maryland, Department of Entomology, College Park, Maryland 20742

ABSTRACT. The possibility of the transovarial transmission of eastern equine encephalitis (EEE) virus by the mosquito *Culiseta melanura* as an overwintering mechanism was investigated in the field and laboratory. Adult female *Cs. melanura* were infected with EEE virus and allowed to oviposit. These mosquitoes were then offered uninfected blood. Eggs from each of the ovarian cycles were allowed to hatch and the larvae reared to adults or pooled in the 3rd or 4th larval stage. No virus was recovered from any F_1 progeny.

Larvae of *Cs. melanura* were collected during the fall and winter months of 1977 and 1978 at the Pocomoke Cypress Swamp in Worcester Co., Maryland. Third and 4th stage larvae were pooled and assayed for EEE virus. Virus was not isolated from the 2,503 larvae processed.

It was concluded that if transovarial transmission of EEE virus is occurring in *Cs. melanura*, it is at a rate of less than 1/2500. This would be such an exceedingly rare event as to have little or no bearing on the overwintering of this virus.

INTRODUCTION

The discovery of the transovarial transmission (TOT) of California group viruses (Watts et al. 1973, LeDuc et al. 1975, McLintock et al. 1976, Barry et al. 1977, Crane et al. 1977, Christensen et al. 1978) and several flaviviruses (Rosen et al. 1978, Aitken et al. 1979) has stimulated speculation that other mosquito-borne viruses may be transmitted in the same manner. Isolations of EEE virus from adult male *Culiseta melanura* (Chamberlain et al. 1961), and field collected larvae (Hayes et al. 1962) have suggested the possibility of TOT. However, sufficient data to implicate TOT as an overwintering mechanism have not been available. In view of the inconclusive data for TOT of EEE virus, a field and laboratory study was undertaken to investigate the possibility that TOT is an overwintering mechanism for the virus.

The Pocomoke Cypress Swamp, in Worcester Co. Maryland, has been shown to be an enzootic focus of EEE virus activity (Moussa et al. 1966, Saugstad et al. 1972, Williams et al. 1972, Dalrymple et al. 1972), and provides suitable breeding sites for *Cs. melanura* (Joseph and Bickley 1969). This swamp was, therefore, selected as the field study area. The laboratory study was conducted in facilities provided by the U.S. Army at the Walter Reed Army Institute of Research (WRAIR).

MATERIALS AND METHODS

FIELD COLLECTIONS. Collections of *Cs. melanura* larvae were made in the Pocomoke Cypress Swamp which is located approximately 5 km southwest of Pocomoke City, Worcester Co., Maryland. The vegetation and substrate of the swamp have been previously described (Moussa et al. 1966, Joseph and Bickley 1969, Saugstad et al. 1972, Williams et al. 1972).

Culiseta melanura larvae are characteristically found in root holes and other well shaded depressions in the swamp substrate. A complete description of these

[1] Part of a dissertation submitted to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree. Scientific Article No. A2844, Contribution No. 5895 of the Maryland Agricultural Experiment Station, Project H-104.

[2] Current address: Yale University School of Medicine, Dept. of Epidemiology and Public Health, 60 College Street, New Haven, CT 06510.

breeding sites is given by Joseph and Bickley (1969). To simulate suitable breeding sites and maximize larval collections, 12 holes were either cut into the swamp substrate or cleared of accumulated debris. Several of these holes were previously used in a study by Williams et al. (1971). The holes were of various sizes and depths, but were in all cases deep enough to reach water. The holes were covered with a 60.96 cm X 60.96 cm piece of plywood with a 20.3 cm X 20.3 cm hole cut in the center. To insure darkness, the center holes were covered with 1 gal (3.79 liter) plastic milk containers painted black and split to allow easy access for sampling. Two natural breeding sites were also sampled.

The breeding sites were examined periodically during 1977. All larvae collected from 15 October through 18 December 1977 were reared to 3rd and/or 4th stage and processed for virus isolation. Larval collections, on a smaller scale, were resumed in March 1978 prior to the first adult emergence, and again in October and December 1978. At the laboratory, larvae were held in an insectary at 25°C and 75% relative humidity with a 16 hr light: 8 hr dark photoperiod. The larvae were maintained in swamp water periodically supplemented with distilled water. Samples were identified, given three distilled water rinses, and pooled in groups of 25 in 2 ml of medium. The medium was composed of Medium 199 Hank's salts (Microbiological Assoc., Walkerville, Md.) supplemented with 20% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), 0.75% sodium bicarbonate (Hem Research Inc., Rockville, Md.), 400 units/ml penicillin and 400 μg/ml streptomycin (Pfizer Laboratories, New York, N.Y.). The pools were either placed on dry ice or transported immediately to WRAIR and placed in a mechanical freezer at −70°C.

VIRUS. The seed virus used in this study was kindly supplied by Dr. A. J. Main, Jr., Yale University School of Medicine, Dept. of Epidemiology and Public Health, New Haven, Ct., as lyophilized 10% suckling mouse brain in 7.5% bovine plasma albumin. The virus, strain 70357, was isolated from *Cs. melanura* collected in Connecticut in 1970, and had undergone one chick and three suckling mouse passages.

The kinetics of virus replication in 1-day-old white leghorn chicks was determined. White leghorn chicks (Truslow Farms, Chestertwon, Md.) were used within 24 hr of hatching. Chicks were inoculated intramuscularly with 0.05 ml of a 10^{-3} dilution of seed virus containing 7.25 X 10^9 PFU/ml. Three different chicks were bled by cardiac puncture every 3 hr for 21 hr. Sera were harvested and stored at −70°C until assayed for virus. At 21 hr all chicks were exsanguinated and the serum harvested and pooled to serve as a virus source.

MOSQUITOES. A colony of *Cs. melanura*, originally colonized in Connecticut (Wallis and Whitman 1969), was established at the University of Maryland, College Park. White leghorn chickens served as a blood source for female mosquitoes. Cotton soaked with 10% sugar water served as a carbohydrate source.

In each of 3 experiments, 100 female *Cs. melanura* were selected at random from the colony cage and placed in specially designed holding containers in groups of 5 or fewer. Mosquitoes were offered infected chicks for a period of 9 to 12 hr. Engorged mosquitoes were isolated individually and allowed to oviposit. Surviving mosquitoes were offered uninfected blood 14 days later. Chicks were bled and the sera harvested for virus assay.

Eggs obtained from infected females were allowed to hatch. F_1 larvae and adults were processed for virus isolation.

VIRUS ISOLATION. A) Mice—Mice of the ICR strain were obtained from the Walter Reed Colony at Forest Glen. Each of 8 suckling mice, 1–3 days old, was inoculated intracerebrally with 0.025 ml of a mosquito pool (Sudia and Chamberlain 1967). Moribund and dead mice were harvested and stored frozen at −70°C.

B) Cell Culture—Baby hamster kidney (BHK-21) cell cultures were used to titrate virus samples by plaque assay. Samples were examined in 10-fold serial dilutions. Each of 2 plaque flasks was inoculated with 0.2 ml of a dilution. These were incubated at 35°C for 1 hr after which each flask was overlaid with 5.0 ml of a medium containing 0.9% agarose (Marine Colloids, Rockland, Me.), 10% 10X Medium 199 (without phenol red and sodium bicarbonate), 150 units/ml penicillin, 150μg/ml streptomycin, and 2.15 mg/ml sodium bicarbonate. The agarose was prepared in double distilled water, autoclaved, and cooled to 42°C at which time the remaining constituents were added. The flasks were incubated inverted at 35°C for 2 days. On the 2nd day the flasks were stained with 0.5 ml of a 2nd overlay medium. This medium contained 1% Difco agar (Difco Laboratories, Detroit, Michigan), Hank's basic salt solution (without phenol red and sodium bicarbonate), 4% 1:300 neutral red (Grand Island Biological Co., Grand Island, N.Y.), 100 units/ml penicillin, and 100μg/ml streptomycin. The overlay was allowed to solidify in the dark, and the flasks inverted and incubated at 35°C. Plaques were counted and recorded the following day.

Two randomly selected virus isolations were identified by plaque reduction neutralization test (PRNT) in BHK-21 cell monolayers. The dilution of virus giving 100 plaque-forming units per 0.2 ml was determined and used in the test. Hyperimmune mouse ascitic fluid (kindly supplied by Dr. J. M. Dalrymple, Virus Dept., WRAIR, Washington, D.C.) prepared against EEE virus was inactivated at 56°C for 30 min and then 2-fold serial dilutions were made. Equal volumes of serum and virus were combined and incubated at room temperature for 1 hr. Plaque flasks were inoculated and handled as previously described.

RESULTS

FIELD DATA. A total of 103 pools, representing 2,503 individuals, were inoculated into suckling mice (Table 1). Virus was not recovered from any of these larvae.

LABORATORY DATA. Assay of the sequential blood samples taken from infected chicks revealed a classic animal virus growth curve. The titers initially declined then increased, reaching a maximum (9–10 $\log_{10}$PFU/ml) between 9 and 12 hours post inoculation. The virus titers remained at near maximum levels for the duration of the 21 hr.

A total of 168 F_1 progeny derived from the initial infective blood meal were processed for virus isolation. These were tested as adults (40 females, 61 males), larvae (62), and pupae (5). Virus was not detected.

Of the 9 females which were offered uninfected blood 14 days after the infective meal, 5 engorged. Blood samples taken from 4 of the chicks fed upon by the mosquitoes produced plaques in BHK-21 cells indicating infection when inoculated into suckling mice.

Two of the 5 mosquitoes oviposited. Eggs in 1 raft did not hatch after 3 days and were subsequently frozen at −70°C for inoculation into mice. Virus was not detected.

Eggs in the remaining raft hatched, and larvae were allowed to develop to adults. The 18 female *Cs. melanura* de-

Table 1. *Culiseta melanura* larvae collected in the Pocomoke Cypress Swamp during 1977 and 1978 and processed for virus isolation.

No. tested	Date collected	Results
848	10/15/77	Negative
335	10/22/77	Negative
48	11/05/77	Negative
551	11/20/77	Negative
103	11/27/77	Negative
170	12/11/77	Negative
43	12/18/77	Negative
17	03/16/78	Negative
48	04/01/78	Negative
57	10/29/78	Negative
283	12/03/78	Negative
Total 2,503		

rived from this 2nd ovarian cycle were isolated and offered uninfected blood. Transmission of the virus was not established. Virus was not recovered from the 18 females, 15 males, or 1 larva processed.

Two virus isolates obtained from the initially infected female mosquitoes were identified as EEE virus by PRNT.

DISCUSSION

Field collections of *Cs. melanura* larvae were conducted at the Pocomoke Cypress Swamp, which has been shown to be an enzootic focus of EEE virus activity. None of the 2,503 individual larvae tested was found infected, at least at detectable levels. This extends the results of a prior study in which 302 larvae were tested and found negative for virus (Muul et al 1975). The failure to detect virus in *Cs. melanura* larvae was reported by Morris and Srihongse (1978) who collected data during an epizootic of EEE virus in New York State. In that study, published during the time the studies described herein were being conducted, the observed minimum field infection rates for non-blooded females were as high as 1/90 in some weeks. Virus was not recovered, however, from larvae, 1st brood adults, or males collected at the same time as non-blooded females which yielded 12 EEE virus isolates.

In the laboratory study, *Cs. melanura* were infected by feeding on viremic chicks in an effort to simulate the natural cycle of EEE virus. A rather high mortality rate was observed, however, among these engorged mosquitoes. Among the ca. 30% of the mosquitoes that engorged, a mortality rate as high as 90% was observed. Such a mortality rate was not observed in the main colony at the University of Maryland. Overall population size did, however, decrease during the winter months despite the relatively constant conditions of the insectary. The deaths of the engorged experimental mosquitoes could not be explained by this annual population crash. It is more likely that the less stable conditions at WRAIR, as noted by large fluctuations in temperature, contributed significantly to these deaths. In addition, the transfer of mosquitoes from one cage into another by aspiration could have traumatized some of these individuals and caused some mortality.

One hundred percent of the mosquitoes which laid fertile eggs were infected as demonstrated by the recovery of virus from the mosquito and/or from blood samples taken from previously uninfected chicks fed upon by these mosquitoes. However, no EEE virus was recovered from either the F_1 progeny or from previously uninfected chicks fed upon by the F_1 females.

LaMotte (1960) and Thomas (1963), working with Japanese encephalitis and western equine encephalitis viruses respectively, demonstrated that virus titers in the ovaries increased with time. Eggs from 2nd or 3rd ovarian cycles should therefore be more likely to contain virus. EEE virus was not isolated, however, from any F_1 progeny produced from a 2nd ovarian cycle. These results strongly suggest that TOT of EEE virus in *Cs. melanura* is not occurring.[3] If TOT of EEE is occurring, it must be at a rate of less than 1/2500 which is certainly not analogous to that for the CAL group of Bunyaviruses. This would be such a rare occurrence as to have little or no bearing on the overwintering of the virus. Recent work by Aitken et al. (1979) has indicated that TOT can occur in *Ae. aegypti* infected with yellow fever (YF) virus. However, these investigators observed low filial infection rates (1/922 and 1/593). If such a phenomenon is occurring with EEE virus, greater numbers of mosquitoes and perhaps an assay system similar to that used by Aitken et al. (1979) should be examined.

ACKNOWLEDGMENT

I am sincerely grateful to Dr. William E. Bickley and Dr. D. M. Watts for estab-

[3] It is recognized that the number of experimental mosquitoes was relatively small.

lishing the program under which this study was conducted. Thanks are also due Maj. Charles L. Bailey for obtaining the laboratory space at WRAIR, and the United States Army for providing me the opportunity to conduct this research.

Special thanks are due Dr. Joel M. Dalrymple and Maj. Charles L. Bailey who acted as my advisors at WRAIR, without whose help this study could not have been completed.

I am sincerely indebted to the University of Maryland Chapter of Sigma Xi for their generous financial support.

Last, but not least, special thanks are due Ms. Wanda Williams for her skilled assistance in the maintenance of the cell cultures used in this study.

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