ARTICLES

TRANSMISSION OF EASTERN EQUINE ENCEPHALITIS VIRUS BY *AEDES AEGYPTI* INFECTED BY LARVAL EXPOSURE AND MEMBRANE FEEDING

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Previous studies on the passage of virus through developing stages of mosquitoes have been made by Whitman and Antunes (1938) using *Aedes aegypti* and yellow fever virus, and by Hodes (1946) using *Culex tritaeniorhynchus* and Japanese encephalitis virus. Additional studies by Collins (1962, 1963) have shown that larvae of *A. aegypti* and *C. quinquefasciatus* mosquitoes could be infected with St. Louis encephalitis virus and that the resultant adult mosquitoes could transmit the virus to baby chicks. Peleg (1965) infected larvae of *A. aegypti* mosquitoes with four group A (eastern equine encephalitis, western equine encephalitis, Venezuelan encephalitis and Sindbis) and four group B (West Nile, Japanese B encephalitis, St. Louis encephalitis and dengue) viruses. All the viruses were subsequently transmitted by bite to suitable experimental animals. In addition, *Culex molestus* mosquito larvae were infected with eastern equine encephalitis and West Nile viruses with subsequent transmission by adult feeding. The infection of adult *A. aegypti* mosquitoes with eastern equine encephalitis virus using a membrane feeding technique has been reported by Collins *et al.*, (1965).

In the present investigation, *A. aegypti* mosquitoes were infected with eastern equine encephalitis virus by both larval exposure and membrane feeding to determine the effect of such multiple infection on the transmission of the virus to baby chicks.

MATERIALS AND METHODS. The eastern equine encephalitis virus (EEE strain NJO-60), was obtained through the courtesy of Dr. Telford Work, Communicable Disease Center, Atlanta, Georgia.

The *A. aegypti* mosquitoes (CD strain) were originally from Technical Development Laboratories, Communicable Disease Center, Savannah, Georgia.

Three groups of mosquitoes were used: (1) those given larval exposure to EEE virus only, (2) those infected with EEE virus as adults only and (3) those given larval exposure and subsequently infected as adults.

The virus suspension for the mosquito larval exposure was prepared by grinding the brains of 15 dead or moribund mice in six milliliters of Bacto heart infusion broth (Difco). After centrifugation for 15 minutes at 1500 r.p.m., the supernatant was added to 100 milliliters of the broth.

Third and fourth instar larvae were immersed in the EEE virus suspension for 4 hours. The larvae were then passed through a 30-minute "wash" in distilled water prior to being returned to the rearing pans. Incubation of the mosquitoes during and after exposure was at 25° to 26° C. in a bacteriological incubator.

Adult mosquitoes were infected by allowing them to feed through a Baudruche (untreated) membrane on serial 10-fold dilutions of EEE virus in fresh heparinized rabbit blood. The brains of six moribund mice previously inoculated with EEE virus were ground in four milliliters of broth and centrifuged for 15 minutes at 1500 r.p.m. One part of the supernatant was added to five parts of defibrinated rabbit blood. This was warmed to 37° C and placed on the membrane which formed the bottom of a ½ pint feeding

cup. The mosquitoes were allowed to feed directly through the screening of their holding cage into the feeding cup. The feeding period was 15 minutes, after which time the engorged mosquitoes were transferred to holding cages and stored in the incubator at 25° to 26° C. The mosquitoes were fed 5 percent Karo solution daily on a cotton pledget.

After 3, 7, 10, 17 and 24 days of extrinsic incubation (after adult infection), mosquitoes were allowed to feed individually on wet baby chicks. Approximately 24 hours later, blood samples were taken by cardiac puncture and the blood inoculated intracerebrally into five mice. Presence of virus in the chick blood as evidenced by the death of the mice from EEE virus constituted evidence of virus transmission by the mosquito.

Samples of adult mosquitoes were collected and killed by freezing immediately after initial feeding and after varying periods of extrinsic incubation. These were stored in a mechanical freezer at —65° to —70° C until titrated. To determine virus titers, mosquitoes were ground individually in a mortar with a one milliliter aliquot of broth containing 1000 units of penicillin and two milligrams of streptomycin per milliliter. The suspension was centrifuged for 15 minutes at 1500 r.p.m. and serial 10-fold dilutions were made in the broth. Five 3-week old mice were inoculated intracerebrally per dilution and the LD_{50}s calculated by the method of Reed and Meunch (1938).

RESULTS. The infection and transmission rates of EEE virus for *A. aegypti* mosquitoes exposed to the virus as larvae only are shown in Table 1. The results have been separated to indicate the effect of the length of the prepupal period (post larval exposure to virus) on the subsequent infection and transmission of the virus. It is apparent that a period of 3 to 4 days as larvae subsequent to exposure resulted in higher transmission rates than did periods of 2 to 3 or 4 to 5 days. A total of 13 transmissions of EEE virus was obtained with mosquitoes exposed as larvae to the virus.

The infection and transmission rates of EEE virus for the mosquitoes infected by the feeding of adult mosquitoes using the membrane feeding technique are presented in Table 2. It is apparent that there was very little transmission of EEE virus (20 percent) through 10 days post-infection. However, on days 17 and 24, the trans-

TABLE 1.—Infection and transmission rates of EEE virus for *Aedes aegypti* mosquitoes exposed as larvae to the virus, presented in relation to the days spent as larvae subsequent to virus exposure.

Days Post Exposure	Positive Mosq./ Total Tested	Transmissions/ Attempts	Positive EEE Virus Titers * (Mouse 1/Log_{10} IC LD_{50})
		2 to 3 Days as Larvae	
18	2/10	0/10	5.0, 3.5
21	5/20	2/20	6.3, 5.4, 5.1, 5.0, 5.0
28	2/10	0/10	4.9, 4.8
		3 to 4 Days as Larvae	
14	3/10	1/10	7.0, 6.5, 6.0
18	3/10	2/10	6.3, 6.3, 5.7
21	5/20	4/20	5.3, 5.2, 5.0, 4.9, 4.9
28	3/20	2/20	5.3, 4.5, 4.5
35	1/10	1/10	5.3
		4 to 5 Days as Larvae	
14	1/10	1/10	6.5
18	1/10	0/10	5.7
21	1/20	0/20	5.7
28	0/10	0/10	...

* Underlined titers are those of mosquitoes which transmitted EEE virus to baby chicks.

TABLE 2.—Infection and transmission rates of EEE virus for *Aedes aegypti* mosquitoes infected by membrane feeding

Days Post Exposure	Positive Mosq./ Total Tested	Transmissions/ Attempts	Positive EEE Virus Titers * (Mouse 1/Log_{10} IC LD_{50})
0	10/10	.....	7.9, 7.3, 7.3, 7.2, 7.0, 6.9, 6.7, 6.7, 6.1, 6.1
3	7/10	1/10	5.8, 5.3, 5.2, 5.2, 5.0, 5.0, 5.0
7	7/10	0/10	6.9, 6.3, 6.3, 6.0, 5.7, 5.5, 5.2
10	7/10	2/10	6.8, 6.2, 6.0, 5.9, 5.8, 5.8, 5.1
17	19/20	16/20	7.2, 7.0, 7.0, 6.9, 6.9, 6.9, 6.7, 6.5, 6.3, 6.3, 6.3, 6.3, 6.2, 6.1, 6.1, 6.0, 6.0, 5.5, 5.5
24	10/10	8/10	6.7, 6.5, 6.5, 6.2, 6.0, 5.9, 5.9, 5.7, 5.7, 5.3

* Underlined titers are those of mosquitoes which transmitted EEE virus to baby chicks.

mission rate had risen to the 80 percent level.

The infection and transmission rates of EEE virus for mosquitoes exposed to infection both as larvae and as adults are presented in Table 3. The mosquitoes were those which had been 3 to 4 days as larvae subsequent to virus exposure. It is apparent that in the period of 3 to 10 days post adult feeding there was an increase in the transmission of EEE virus as a result of the dual routes of exposure. With larval exposure only, those mosquitoes which had been 3 to 4 days as larvae subsequent to exposure had a transmission rate of 7 out of 40 (18 percent). With the adult infection only, the transmission rate was 3 out of 30 (10 percent). With the dual routes of exposure, the transmission rate was 14 out of 30 (47 percent). The transmission rates on days 17 and 24 post adult feeding were intermediate between those obtained with larval exposure and those obtained from adult infection only.

DISCUSSION. Previous studies (Collins, 1962) had indicated that *A. aegypti* mosquitoes could be infected with St. Louis

TABLE 3.—Infection and transmission rates of EEE virus for *Aedes aegypti* mosquitoes infected by larval exposure and by membrane feeding.

Days Post Larval	Exposure Adult	Positive Mosq./ Total Tested	Transmissions/ Attempts	Positive EEE Virus Titers* (Mouse 1/Log_{10} IC LD_{50})
14	3	9/10	3/10	6.0, 6.0, 5.5, 5.3, 5.2, 5.0, 4.9, 4.9, 4.8
18	7	8/10	5/10	7.1, 6.2, 6.0, 6.0, 5.9, 5.7, 5.0, 4.9
21	10	9/10	6/10	7.3, 7.0, 6.8, 6.3, 6.2, 6.0, 6.0, 5.0, 5.0
28	17	8/10	5/10	7.8, 7.2, 7.0, 6.5, 6.3, 6.2, 5.9, 5.9
35	24	9/10	4/10	6.5, 6.5, 6.5, 6.3, 6.3, 5.9, 4.8, 4.7, 4.7

* Underlined titers are those of mosquitoes which transmitted EEE virus to baby chicks.

encephalitis virus by exposure of the larvae to a virus suspension. The present investigation indicates that this is also true for EEE virus.

The addition of a second infection by the adult feeding technique in mosquitoes previously exposed as larvae appeared to result in a population having an early (3 to 10 day) transmission rate higher than could be explained by the addition of the transmission rates for those mosquitoes infected by either route alone. It is possible that the previous larval exposure may have "conditioned" the mosquitoes so that the second infection with EEE virus was more readily transmitted by these mosquitoes. Control lots of larval infected mosquitoes which received blood meals without the EEE virus failed to show any difference in transmission rate from those larval exposed mosquitoes not given an intermediate blood meal. The failure of mosquitoes infected by both routes to attain a transmission level after 17 and 24 days as high as those infected by adult feeding alone again indicates some effect of the previous larval exposure on the transmission of EEE virus by these mosquitoes. An explanation of these results would not appear to be evident at this time.

SUMMARY. Larvae of *Aedes aegypti* mosquitoes were exposed to a mouse brain suspension of eastern equine encephalitis virus (EEE). Thirteen of 160 (8 percent) resultant adult mosquitoes subsequently transmitted the infection to baby chicks.

Mosquitoes infected by membrane feeding on an EEE virus suspension in defibrinated rabbit blood were compared with mosquitoes infected by larval exposure and by both routes of infection. In the period of 3 to 10 days post adult infection, the mosquitoes infected by both routes had a transmission rate of 47 percent as compared with 18 percent for larval infected and 10 percent for adult infected mosquitoes. After 17 to 24 days of extrinsic incubation, the mosquitoes infected by both routes had a transmission rate of 36 percent compared with 10 percent for larval infected and 80 percent for adult infected mosquitoes.

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The annual meeting of the NMCA will be held on Nov. 2, 3 and 4, 1966 at the Hearthstone Motor Inn on Rte. 44, Junction of 114A in Seekonk, Mass. Subjects to be discussed include Pesticide Residues in Soil and Water, Toxicity of Chemicals to Saltmarsh Wildlife, and others.