Eastern equine encephalomyelitis (EEE) virus is a mosquito transmitted virus in the family Togaviridae, genus Alphavirus. Its genome consists of a single strand of RNA with plus polarity and is approximately 12 kb in length. Replication takes place within the cytoplasm of host cells and virions are infectious after they bud through the host cell membrane.

The EEE virus complex consists of two serologic varieties. The North American variety is maintained in enzootic cycles by the mosquito Culiseta melanura, which transmits virus among passerine birds in freshwater swamps in eastern North America. Disease occurs when other mosquito species transfer virus from songbirds to humans, horses, and exotic gamebirds. The South American variety is transmitted among small mammals, occasionally birds, by mosquitoes in the genus Culex subgenus Melanoconion from southern Mexico to northern Argentina. Although transovarial transmission among mosquito vectors has been reported for the closely related western equine encephalomyelitis virus, an assortment of field studies have failed to provide evidence that EEE virus is transmitted vertically (reviewed by Scott and Weaver). Persistence of this virus appears to depend entirely on horizontal transmission, alternating infections between mosquito vectors and vertebrate hosts.

Ultrastructural studies on the infection of EEE virus in Cs. melanura revealed cytopathologic changes in the midgut epithelium of infected mosquitoes. This observation challenged long-standing ideas about the nature of the relationship between arthropod-borne viruses (arboviruses) and their mosquito hosts. The traditional perspective is that this group of taxonomically diverse viruses cause severe disease and pathology in vertebrates but have no detectable detrimental effect on their insect vectors. This way of thinking has been challenged on a theoretical basis.

Materials and Methods

Infection of mosquitoes. The maintenance and care of experimental animals in these studies complied with the National Institutes of Health guidelines for the humane use of laboratory animals. All experiments were conducted with a colony of Cs. melanura that were provided a 5–10% sucrose solution and maintained in environmental chambers at 25°C, 80% relative humidity, and a 16-hr light and 8-hr dark photoperiod. Five to seven-day-old adult female mosquitoes were exposed to virus by feeding on viremic chickens. Groups of mosquitoes were housed in 3.8-liter plastic cages. Individual mosquitoes were held in 0.5-liter cardboard cages.

Chicken hosts were inoculated with 10^9.5 baby hamster kidney cell (BHK) 50% tissue culture infectious doses (TCID50) of EEE virus 6–24 hr prior to a 1-hr exposure to mosquitoes (Table 1). Two-tenths of a milliliter of blood was collected by venipuncture from chickens immediately before and after mosquitoes fed, mixed with 0.9 ml of avian diluent, centrifuged, and stored at –70°C until assayed for virus. Serum was assayed for virus on monolayers of BHK cells to estimate the amount of virus imbibed by mosquitoes. Titers are expressed as log10 TCID50 per 1.0 ml of blood. No virus was detected in blood from any of the control birds. In two experiments a small number of mosquitoes collected within 1 hr after they fed on viremic birds were collected and frozen at –70°C. To demonstrate that they had imbibed infectious virus, frozen specimens were thawed, triturated in mosquito diluent, and virus in each specimen was titrated on BHK cells.
Fecundity. The first experiment was a comparison of the number of larvae that hatched from the first rafts of eggs that were laid by virus-exposed versus control mosquitoes. Fully engorged and previously mated mosquitoes were individually maintained in cages containing water for oviposition. Within 24 hr of when eggs were laid, hatched larvae were counted using a dissecting microscope to confirm egg viability.

Oogenesis. A follow-up experiment was conducted to determine if virus affects oogenesis. Engorged mosquitoes from control and virus exposed groups were collected and frozen at 12-hr intervals for five days. Ovaries were dissected and made into wet mounts on glass microscope slides. Using bright-field microscopy, ovaries were categorized into one of Christophers’ five stages of ovarian development.23

Blood feeding success. The effect of virus on the ability of mosquitoes to obtain a blood meal, a requirement for Cs. melanura to lay eggs,4 was examined by comparing feeding success of infected versus uninfected mosquitoes. After fully engorging, groups of mosquitoes were held for 19 days and then allowed 1 hr to take a second meal from uninfected chicks. The number of fully and partially engorged mosquitoes was recorded. Mosquitoes categorized as empty were dissected and microscopically examined to determine if their posterior midgut contained a small amount of blood that was undetectable by external examination.

Mosquito survival. In a series of experiments the effect of virus on mosquito survival was examined. Mortality among groups of control and infected mosquitoes was monitored daily until all mosquitoes died. No blood meals, other than the initial one, were provided during the observation period.

Life table statistics. The effect of virus on mosquito fitness was examined by comparing life table statistics for virus-exposed versus control mosquitoes. Mosquitoes were allowed to randomly mate and then were exposed to either an EEE virus infected or control chick. Each fully engorged mosquito was held in a separate cage and provided an oviposition substrate. After laying her eggs, each female was provided a 1-hr feeding opportunity each day until she imbibed another blood meal from an uninfected chick. The number of first instar larvae that hatched from her eggs were counted as described in the methods for fecundity studies. This process of offering blood meals, allowing mosquitoes to lay eggs, and counting larvae that hatched from eggs was repeated daily until all mosquitoes died. The methods of Price24 were used to calculate life table statistics.

Statistical analysis. Statistical analyses were done using SAS.25 Survival data, expected number of daughters, reproductive expectation, and net replacement values were compared using a Kruskal-Wallis k-sample test.26 A one-way analysis of variance26 was used to analyze data on blood feeding success and fecundity. Results from the oogenesis study were compared by chi-square analysis.26

RESULTS

Mosquito infection. Virus infection rates for a subsample of mosquitoes from each experiment were not determined because 1) virus assay of whole insects requires killing mosquitoes and would reduce already small sample sizes and 2) determining infection status by removing and titering a leg might have undefined effects, potentially detrimental, on mosquitoes and would compromise the interpretation of data. Even though it is possible that not all mosquitoes in virus-exposed groups became infected, when the titer of EEE virus in avian host blood exceeds $10^{10}$ TCID$_{50}$ per 1.0 ml, as it did in all experimental infections discussed below, results from past experiments show that infection rates of Cs. melanura are 100%.”21-23,30 Viremias in experimental chicken hosts were similar to those previously determined for natural avian hosts.4

Fecundity. Mosquitoes that fed on the control chick produced more larvae ($n = 29$, $x = 93 + 34$ (mean ± SD); degrees of freedom [df] = 1, $F = 5.84$, $P < 0.02$) than did mosquitoes that fed on the virus infected chick ($n = 25$, $x = 73 ± 28$). Titer of virus in the blood of the viremic chick (strain ME-77132) were $10^{6.4}$ before and $10^{6.8}$ after exposure to mosquitoes. All six mosquitoes collected and assayed for virus within 1 hr after feeding on the viremic chick contained detectable amounts of virus. The virus titer of triturated mosquitoes ranged from $10^{4.8}$ to $10^{5.8}$.

Oogenesis. Chi-square analysis by time of collection and stage of ovarian development revealed no significant differences in ovarian development between the two treatment groups ($\chi^2 < 0.20$, $P > 0.50$), except for collections at 120 hr (Table 2). At that time more mosquitoes that fed on the infected chicks had laid more of their eggs than had controls ($\chi^2 = 6.4$, $P < 0.025$). Titer of virus in the blood of the viremic chick (strain ME-77132) were $10^{7.3}$ before and $10^{8.1}$ after exposure to mosquitoes.

Blood-feeding success. Feeding successes of virus exposed and control groups were different (Table 3), whether the analysis was based on mosquitoes that were fully engorged, partially engorged, contained a trace of blood in their midgut or some combination of these three categories of engorgement ($F = 0.12$, $P > 0.74$). Across five paired replicates of 13–63 mosquitoes that fed on different chicks, feeding rates were 67% for virus exposed versus 65% for control mosquitoes. Mean virus titers for blood from five viremic chicks (strain ME-77132) were $10^{4.3}$ before and $10^{4.2}$ after exposure to mosquitoes.

Effect of virus on mosquito survival. When a single strain of EEE virus was studied (ME-77132, Figure 1a), con-

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Passage history*</th>
<th>Isolation location</th>
<th>Year</th>
<th>Host</th>
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<td>1933</td>
<td>Horse</td>
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<td>1977</td>
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<tr>
<td>2061-88</td>
<td>C6/36-1</td>
<td>Maryland</td>
<td>1988</td>
<td>Culiseta melanura</td>
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* sm = suckling mice; Vero = African green monkey kidney cell line; C6/36 = Aedes albopictus cell line.
Virus-5
Virus-4
Virus-3
Virus-2
Virus-1

The number of mosquitoes in each treatment group were as follows: Ten Broeck n = 21, 21; 2061-88 n = 23, 24; and controls n = 20, 21.

Life table statistics. Two groups of 15 randomly mated Cs. melanura fed on a virus infected or on a control chick. Titors of virus in the blood of the viremic chick (strain ME-77132) were $10^{6.8}$ before and $10^{7.6}$ after exposure to mosquitoes. Expected number of daughters ($m_x$, Figure 2b; df = 1, $F = 6.88$, $P < 0.01$), reproductive expectation values ($l_x$, Figure 2c; df = 1, $F = 8.59$, $P < 0.005$), and cumulative reproductive expectation values (net replacement, $\Sigma_1m_x$, Figure 2d; df = 1, $F = 492.66$, $P < 0.0001$) were significantly higher for control mosquitoes than for mosquitoes that imbibed viremic blood. Differences in reproductive output were evident throughout the time that mosquitoes laid eggs (Figure 2d). The net replacement rate for control mosquitoes ($R_o$ = 62) was 82% greater than that of mosquitoes that fed on the viremic chick ($R_o$ = 34). Net replacement values should be viewed in a relative sense because they do not account for mortality of immature forms before they became reproductively competent. There was no difference in age specific survivorship between the two groups ($l_x$, Figure 2a; df = 1, $F = 0.61$, $P > 0.4$). Periodic decreases in survival were associated with oviposition (Figure 2a), indicating that elements connected with laying eggs are a mortality factor for Cs. melanura.

**DISCUSSION**

Results from this study indicate that survival and reproduction of Cs. melanura are reduced after the mosquito imbibes North American strains of EEE virus. Variation in virulence, as determined by significantly different survival, was not detectable among different virus strains isolated over a

<table>
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<tr>
<th>Treatment</th>
<th>Number exposed</th>
<th>Fully engorged</th>
<th>Partially engorged</th>
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<th>Empty</th>
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<tr>
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**TABLE 2**

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<td>36</td>
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<tr>
<td>84</td>
<td>96</td>
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<td>120</td>
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</table>

**TABLE 3**

A comparison of ovarian development in Culiseta melanura that fed on eastern equine encephalomyelitis (EEE) virus (strain ME-77132) infected versus control chicks.
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FIGURE 1. Reductions in survival of *Culiseta melanura* due to infections with eastern equine encephalomyelitis virus. 

a, a single virus strain. b, two virus strains isolated 11 years apart. c, two virus strains isolated 55 years apart.

55-year time span. Virus-induced mortality was only detected when mosquitoes were not given subsequent blood meals and, therefore, were not subject to oviposition-associated mortality. No effect of virus was detected on the ability of mosquitoes to obtain a blood meal or on the rate of oocyte development. Significantly, however, virus-associated reductions in mosquito fecundity and total fitness were observed (Figure 2). Because virus did not affect the rate of oogenesis, it was concluded that the effect on fecundity was associated with variation in the number of oocytes that developed, rather than a delay in reproductive output.

Effects of virus on mosquito survival were not apparent until approximately 14–21 days after imbibing a viremic blood meal (Figure 1). The probability of *Cs. melanura* refeeding on vertebrate blood and transmit virus is highest 7–10 days after imbibing an infected blood meal. Reductions in mosquito survival, therefore, occur after the probability of virus transmission is highest and would not be expected to reduce the probability of virus transmission.

Unlike effects on survival, reduction in fecundity (Figure 2) occurred within days after imbibing viremic blood, indicating that fitness reductions for EEE virus exposed *Cs. melanura* occur early in adult mosquito life and confer a selective disadvantage. Reduction in the number of mosquito progeny would be expected to affect the efficiency of virus transmission only if it reduced the number of potential vectors for virus progeny. This virus is transmitted horizontally and infection rates for wild mosquitoes are low (≤ 3/1,000 tested). Virulence may not have been measurably attenuated because relatively few mosquitoes in the population are virus-infected, infected vectors are not related to one another, and virus-induced reductions in mosquito survival occur after the time when the probability of transmission is highest. Results from experiments described herein did not determine whether virulence is directly related to a virus fitness advantage, but suggest that such a relationship may exist. Additional research is needed to clarify the connection between mosquito virulence and efficiency of EEE virus transmission.

Results from these experiments support previous studies on mosquito-virus interactions and indicate that reproductively successful arboviruses can have deleterious effects on their mosquito vectors. There were measurable reductions in vector fitness (Figure 2) and thus, a selective disadvantage for virus-infected mosquito hosts that are required for EEE virus transmission in North America. In earlier studies, *Aedes triseriatus* that were orally infected with La Crosse virus probed a vertebrate host more and imbibed less blood than uninfected cohorts. Larval *Ae. dorsalis* and *Ae. melanimon* that were vertically infected with California encephalitis virus took longer to develop to adults than did uninfected siblings. *Culex pipiens* that were horizontally infected with Rift Valley fever virus had a reduced ability to obtain a blood meal, to lay eggs, and to survive as adults than uninfected controls. Future investigations of arbovirus-vector interactions should consider 1) that some degree of virulence in vectors may be advantageous for the virus and 2) specifically test the assumption that virulence is associated with a virus fitness advantage.

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Authors’ addresses: Thomas W. Scott, Department of Entomology,
University of California, One Shields Avenue, Davis, CA 95616-8584. Leslie H. Lorenz, Department of Entomology, University of Maryland, College Park, MD 20472.

Reprint requests: Thomas W. Scott, Department of Entomology, University of California, One Shields Avenue, Davis, CA 95616-8584.

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