*Journal of the American Mosquito Control Association,* 20(3):277–282, 2004


# METHODS FOR STUDYING THE VECTOR COMPETENCE OF *CULEX TARSALIS* FOR WESTERN EQUINE ENCEPHALOMYELITIS VIRUS

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ABSTRACT. Female *Culex tarsalis* fed heparinized chicken blood–western equine encephalomyelitis virus (WEEV) mixtures through a biomembrane feeder were compared with females fed sweetened blood–virus mixtures presented in pledgets or as hanging drops or to restrained chickens with natural or artificial viremias. Results indicated that sodium heparin did not adversely affect the infection of *Culex tarsalis* with WEEV. Overall advantages of the biomembrane system included 1) increased blood feeding frequency, 2) control of the infectious virus dose, and 3) greater or similar infection rates and body titers to females taking blood meals from viremic chickens. Anesthetizing females with triethylamine for in vitro transmission assessment using the capillary tube method produced results similar to immobilization using cold or $CO_2$ + cold. Our research provided insight into tools useful to investigate the infection and transmission of WEEV by *Cx. tarsalis.*

**KEY WORDS** Western equine encephalomyelitis virus, *Culex tarsalis,* vector competence, membrane feeder, heparin, triethylamine

## INTRODUCTION

Different laboratories have used a variety of in vivo and in vitro blood feeding techniques to infect mosquitoes with arboviruses to investigate their vector competence, resulting in a range of quantitative estimates potentially affected by blood feeding methods. Our laboratory has blood-fed mosquitoes on gauze pledgets, soaked with defibrinated, sweetened rabbit blood suspensions containing various concentrations of virus to quantitatively evaluate dependence of susceptibility on virus dose (Hardy and Reeves 1990, Kramer et al. 1993, Reisen et al. 1997). Pledgets precluded the need for a live host, such as a 1–3-day-old chicken (chick), and allowed control of the virus titer in the infectious blood meal. Recently, we evaluated the vector competence of a number of mosquito species to West Nile virus (WNV) by feeding them on droplets of sweetened, defibrinated rabbit blood containing WNV (Goddard et al. 2002). However, defibrinated blood does not clot normally in the midgut, resulting in fewer virions coming in direct contact with the receptor sites on the microvilli than when the blood meal clots normally (Weaver et al. 1993). Alternatively, blood can be treated with heparin to preclude clotting, but little has been reported about the effects of heparin on western equine encephalomyelitis virus (WEEV) infection in mosquitoes. Heparin reputedly reduces cell infection with Flaviviruses (Hurrelbrink and McMinn 2001; Lee and Lobigs 2002); however, there were no differences in infection of mosquitoes when Rift Valley Fever virus (*Phlebovirus*) was mixed with defibrinated or heparinized blood and presented on pledgets (Turell 1988). Historically, transmission ability of mosquitoes was evaluated by using a receptor host such as a chick (Hardy and Reeves 1990). Recently, an in vitro capillary tube method (Aitken 1977) has been used with comparable results (Cornel and Jupp 1989). Using the in vitro method, mosquitoes have been immobilized using different protocols including $CO_2$, cold, and triethylamine (Hardy and Reeves 1990, Goddard et al. 2002). Triethylamine would seem useful for vector competence studies because females remain immobile and alive for long time periods after being anesthetized and this compound does not reduce virus titer (Kramer et al. 1990, O'Guinn and Turell 2002).

We have begun to quantitatively evaluate the effects of WEEV (*Togaviridae, Alphavirus*) dose on *Culex tarsalis* Coquillett vector competence, pathology, and fitness and have switched to a biomembrane feeding apparatus to enhance mosquito blood feeding rates while retaining control of the infectious virus dose. Prior to these experiments, we evaluated the impact of blood treated with sodium heparin on the mosquito feeding response and virus infection. The present paper summarizes experiments comparing methods for infecting *Cx. tarsalis* with WEEV and immobilizing females for transmission attempts.

## MATERIALS AND METHODS

### Mosquitoes

We used the high virus producer (HVP) susceptible *Cx. tarsalis* colony (Hardy and Reeves 1990) and the Bakersfield Field Station (BFS) colony established in 1953 (Bellamy and Kardos 1958). The HVP strain recently was reselected for 3 generations by feeding on 5 $\log_{10}$ plaque forming units

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(PFU) of WEEV/ml and selecting for females amplifying virus to >5 $\log_{10}$ PFU per female. Progeny of these females were combined to produce the colony used in the present study. Mosquitoes were reared by standard methods in an insectary that was maintained at 24 ± 2°C and a photoperiod 14:10 (L:D) h (Mahmood 1999). Emerging adults were aged for 4–6 days and were provided 10% sucrose solution until 24 h before experimentation. After each blood feeding trial, fully engorged females were sorted and then maintained at 26°C in pint cartons, where they were offered fresh 10% sucrose on cotton pads daily. Virus infection was determined for up to 20–25 surviving females per treatment group. The ability of females to transmit virus per os was determined using the capillary-tube method (Aitken 1977), with secretions collected in a 1:1 by volume solution of fetal calf serum and 10% sucrose and then expelled into 300 µl of virus diluent (phosphate buffered saline plus 20% fetal bovine serum and antibiotics [100 U penicillin, 100 U streptomycin, 200 U nystatin]).

## Virus

The BFS1703 strain of WEEV, originally isolated from *Cx. tarsalis* collected near Bakersfield in 1953, has been used as a reference strain in our laboratory and was at suckling mouse passage 2 and Vero cell passage 1 when used in the current experiments (Hardy and Reeves 1990). Virus was stored at −80°C until thawed in ice water for experimentation. Mosquitoes were exposed to suspensions of stock virus (7 $\log_{10}$ PFU/ml) diluted in heparinized or defibrinated blood and presented in glass feeders covered with a sausage casing biomembrane made from hog intestines (sausage casings packed in brine purchased from local grocery stores) and heated by a water circulation system to 37°C (Rutledge et al. 1964). When presented on pledgets or as hanging drops at room temperature, a separate blood virus suspension was sweetened by the addition of sucrose diluted to a 2.5% final volume to ensure the passage of the blood meal into the midgut (Hardy and Reeves 1990). Chickens were used to infect mosquitoes by two methods.[2] Approximately 2-day-old chicks were infected by subcutaneous inoculation in the cervical area and held for 1 day prior to being restrained without anesthesia on the screened tops of cartons. Alternatively, chicks were injected intravenously with virus through the jugular vein and then exposed to mosquitoes within 15 min (Weaver et al. 1993). Viremia was monitored before and after mosquito feeding by taking 0.1 ml whole-blood samples via jugular puncture, diluting blood 1:5 in virus diluent, and immediately freezing at −80°C. Mosquitoes were exposed to all treatments for ≤1 h; however, when most blood fed during a shorter time interval, that treatment was removed and the mosquitoes sorted.

## Virus testing

The quantity of virus present in mosquito bodies and salivary gland secretions, blood virus suspensions, and chicken blood samples was measured by plaque assay (Reisen et al. 1993) using Vero cells cultured in 6- or 12-well plates (Costar, Corning, NY) in 1× modified Eagle medium growth medium supplemented with 7.5% sodium bicarbonate solution and incubated at 37°C with 5% $CO_2$. Confluent monolayers of cells were inoculated with 0.1 ml of sample solution and adsorbed at 37°C in 5% $CO_2$ for 1.5 h. After adsorption, a single overlay containing neutral red was applied and the plates read after 4 days.

## Experimental design

The rationale and objectives for each of our 4 experiments are summarized below.

Experiment 1 compared the effects of host blood type (rabbit versus chicken) and anticlotting method (defibrination versus heparin) on the virus infection rate and resulting body titer of *Cx. tarsalis* females. Freshly collected blood was shaken with 3-mm-diameter glass beads for 20–30 min for defibrination and compared concurrently with blood collected into commercial 10-ml green-top vacutainer tubes containing sodium heparin (143 freeze-dried USP units per tube, Becton-Dickson, Franklin Lakes, NJ).

Experiment 2 compared infection in *Cx. tarsalis* females after feeding on a chick infected 1 day previously by subcutaneous syringe inoculation or on heparinized chicken blood–virus mixtures presented in a biomembrane feeder, on gauze pledgets, or as hanging droplets. Blood virus samples were taken before and after mosquito feeding, and in some trials, blood-fed mosquitoes were frozen within 15 min of feeding to determine the titer of virus imbibed within the blood meal. Experiment 2 was repeated thrice.

Experiment 3 evaluated the immediate method of producing an artificial viremia in chicks (Weaver et al. 1993) because of variation in the titer of the delayed viremia among chicks in experiment 2. In a preliminary experiment, 6 chicks were infected by intravenous inoculation of stock virus into the jugular vein. One chick was bled at each of six 15-min intervals postinoculation to determine virus persistence in the blood; all chicks were rebled at the close of the experiment. In the principle experiment, mosquitoes were offered restrained chicks 15–45 min after being inoculated with three 100-fold dilutions of WEEV and compared with mos-

[2] The use of chickens for vector competence studies was approved under Protocol 9609 by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis.

Table 1. Effects of blood source and treatment on engorgement and WEEV infection in female *Culex tarsalis*.

| Host[1] | Treatment[2] | Virus[3] 0 | Virus[3] 1 h | Fed[4] (%) | Infected[5] (%) | Infected[5] Titer |
|---|---|---|---|---|---|---|
| Rabbit | Def | 5.5 | 5.3 | 85 | 60 | 4.7 |
| Rabbit | Hep | 5.2 | 4.5 | 65 | 65 | 4.7 |
| Chicken | Def | 5.1 | 4.5 | 87 | 70 | 4.0 |
| Chicken | Hep | 5.0 | 4.5 | 90 | 80 | 4.8 |

[1] Freshly drawn blood source.
[2] Blood treatment: defibrinated (Def), shaken for 20 min with glass beads; heparinized (Hep), blood taken into standard heparinized vacutainer.
[3] Blood virus titer ($\log_{10}$ PFU/ml) in membrane feeder before and after mosquito feeding.
[4] Percent of 40 females that blood fed.
[5] Percent of 20 engorged females that were infected and mean virus titer in infected females ($\log_{10}$ PFU/female).

quitoes offered similar titers of virus mixed in heparinized chicken blood and presented in a membrane feeder.

Experiment 4 compared different methods of mosquito manipulation to assess transmission. Females were infected by feeding on a WEEV–heparinized chicken blood mixture in a biomembrane feeder and then were incubated for 8–14 days, after which transmission was attempted by the capillary method using mosquitoes that were 1) anesthetized lightly with $CO_2$ gas and then chilled in a test tube on wet ice (standard method), 2) chilled on wet ice, and 3) anesthetized with triethylamine (TEA). Triethylamine, used routinely to immobilize field samples for virus testing, was shown not to reduce virus titer in infected mosquitoes (Kramer et al. 1990, O'Guinn and Turell 2002) and was used in our recent transmission studies (Goddard et al. 2002).

## RESULTS

### Experiment 1: Blood source and treatment

Titers in blood–virus mixtures added to the biomembrane feeders ranged from 5 to 6 $\log_{10}$ PFU/ml and decreased slightly during the subsequent hour at 37°C (Table 1). When tested by chi-square, the percentage of females blood feeding and infected with WEEV did not differ significantly ($P > 0.05$) between blood sources (rabbit or chicken) or treatments (defibrination or heparin). When tested by a 2-way analysis of variance (ANOVA, [Hintze 1998]), mean titers of WEEV in positive females did not vary significantly ($P > 0.05$) between blood source or treatment.

### Experiment 2: Mosquito infection methods

During trial 1, significantly more BFS colony females blood fed on artificial viral presentations (24%, $n = 180$) than on the 2 chickens (11%, $n = 120$, $\chi^2 = 7.8$, df = 1, $P = 0.005$); however, infection rates were variable (Table 2). In trial 2, we used the HVP colony previously selected for WEEV susceptibility. Although infection rates remained variable (Table 2), highest infection rates

Table 2. Comparison of 4 methods for infecting mosquitoes per os with WEE virus.

| Infection method | Virus dose[1] | Titer per blood fed mosq.[2] | $n$[3] | Infected (%)[4] | Titer per female[5] |
|---|---|---|---|---|---|
| Trial 1, BFS colony[6] | | | | | |
| Chick 1 | 4.0 | 1.1 | 5 | 0 | |
| Chick 2 | 3.5 | 1.3 | 8 | 88 | 5.3 a |
| Membrane | 5.8 | 2.8 | 15 | 67 | 4.9 a |
| Pledget | 5.1 | 3.2 | 14 | 0 | |
| Hanging drop | 5.1 | 3.3 | 15 | 0 | |
| Trial 2, HVP colony | | | | | |
| Membrane | 5.3 | nd | 20 | 85 | 3.9 a |
| Pledget | 5.0 | nd | 20 | 5 | 4.4 a |
| Hanging drop | 5.0 | nd | 20 | 0 | |
| Trial 3, HVP colony | | | | | |
| Chick 1 | 6.7 | 3.6 | 25 | 100 | 5.1 a, b |
| Chick 2 | 7.0 | 3.7 | 25 | 100 | 5.3 a |
| Membrane | 5.3 | 2.6 | 25 | 80 | 4.5 a, b, c |
| Pledget | 5.5 | 2.5 | 31 | 61 | 3.8 c |
| Hanging drop | 5.5 | 2.6 | 25 | 52 | 4.3 b, c |

[1] Virus exposure dose in $\log_{10}$ PFU/ml.
[2] Mean virus titer ($\log_{10}$ PFU/mosquito) in 3–5 blood engorged females frozen within 1 h of feeding.
[3] Number blood feeding ($n = 60$ females) and surviving to be frozen at 5 days postinoculation at 26°C ($n = 20$–31 tested per group, extras discarded).
[4] Percent of those tested that were infected.
[5] Virus titer ($\log_{10}$ PFU/mosquito) in infected females; means within trials not significantly different ($P > 0.05$) when followed by the same letter using a least significant range test.
[6] BFS, Bakersfield Field Station; HVP, high virus producer.

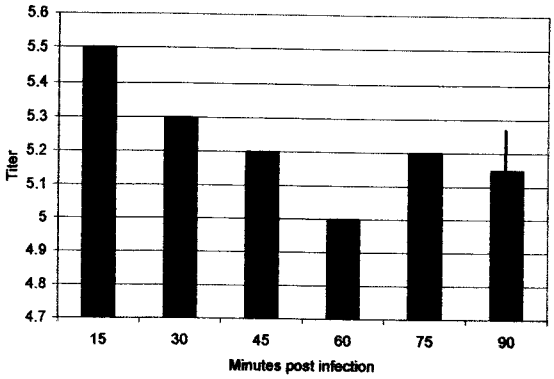


Fig. 1. WEEV titer ($\log_{10}$ PFU/ml) in blood samples taken by jugular puncture from 1-day-old chicks plotted as a function of time in minutes after inoculation. The 90-min sample was the average (+ SD) of a 2nd sample taken from all 6 chicks.

were obtained for females that engorged on western equine encephalomyelitis virus suspensions from the biomembrane feeder. In trial 3, infection rates were significantly greatest ($\chi^2 = 29.9$, df = 4, $P < 0.001$) for those females that fed on chickens with viremia $\geq 6$ $\log_{10}$ PFU of WEEV/ml (Table 2). The mean WEEV titer per infected female varied significantly (F = 3.68, df = 4,97, $P < 0.01$) among groups, being greatest for females that fed on chickens and not different among females feeding on artificial blood meals.

### Experiment 3. Use of chickens immediately after intravenous inoculation

Failure to consistently produce elevated viremias in chicks 1 day postinoculation during experiment 2 led us to evaluate an artificial viremia method, where mosquitoes were exposed to chicks immediately after intravenous inoculation. In a preliminary evaluation, there was little change in WEEV titer in blood samples taken from 1 of 6 chickens at 15-min intervals for 90 min after intravenous inoculation (Fig. 1), thereby providing sufficient time to complete mosquito infection studies.

We then compared *Cx. tarsalis* engorgement and infection when females were offered chickens with an artificial viremia or a heparinized chicken blood–virus mixture in a biomembrane feeder (Table 3). Overall, significantly ($\chi^2 = 31.9$, df = 1, $P < 0.001$) more females engorged on the biomembrane feeders (96%, $n = 90$) than on the infected chickens (61%, $n = 120$), but there were no differences ($P > 0.05$) among infection rates in mosquitoes exposed to comparable titers (Table 3). A 2-way ANOVA compared mean virus titers of infected females after feeding on chickens or the biomembrane feeder. Overall, mean titer for 47 females infected after feeding on the biomembrane feeder (3.7 $\log_{10}$ PFU/mosquito) was not significantly different (F = 2.14, df = 1,72, $P = 0.15$) from the mean titer for 31 females infected after feeding on the chickens (4.4 $\log_{10}$ PFU/mosquito). Mean mosquito body titer decreased significantly as a function of virus titer in chicks, but not in the biomembrane (Table 3), as indicated by significant interaction effect in the ANOVA (F = 3.39, df = 2,72; $P = 0.03$).

### Experiment 4. Effects of immobilization methods on transmission

Female *Cx. tarsalis* were infected by engorging on a mixture of WEEV and heparinized blood in the biomembrane feeder, held for 8, 12, or 14 days at 26°C, and then immobilized by three different methods prior to transmission attempts using the capillary-tube method. There were no significant differences in mean body titer among females in the three transmission groups (range = 3.5–4.0 $\log_{10}$ PFU/mosquito, $P > 0.1$); however, titer increased significantly with holding time (F = 5.3, df

Table 3. Comparison of *Culex tarsalis* engorgement and virus infection after feeding on 3 dilutions of WEEV presented in a chicken used immediately after intravenous inoculation or in a biomembrane feeder containing a virus–heparinized chicken blood mixture.

| Virus dilution | Method[1] | Titer[2] | Mosq. titer[3] | Engorged[4] (%) | Infected[5] (%) | Infected[5] Titer |
|---|---|---|---|---|---|---|
| 1:10 | Chicken | 6.2 | 3.4 | 88 | 90 | 4.8 |
| | Membrane | 6.3 | 4.3 | 63 | 95 | 3.7 |
| 1:1000 | Chicken | 4.0 | 1.7 | 25 | 86[6] | 4.6 |
| | Membrane | 4.6 | 2.6 | 100 | 90 | 3.5 |
| 1:100,000 | Chicken | 3.6 | 1.2 | 75 | 35 | 3.1 |
| | Membrane | <1.2 | 1.2 | 100 | 50 | 3.8 |

[1] Method; mosquitoes feeding on chicken immediately after intravenous inoculation or on heparinized chicken blood in a biomembrane feeder.
[2] Titer of virus in blood ($\log_{10}$ PFU/ml) after mosquito feeding taken by jugular puncture of the chick or from membrane feeder.
[3] Mean titer of virus in 3 mosquitoes immediately after engorgement.
[4] Percent of females blood feeding on chick ($n = 40$/group) or biomembrane ($n = 30$/group).
[5] Percent of females infected after incubation ($n = 20$ tested/group) and mean titer of virus in positive females.
[6] $n = 7$ females.

Table 4. Number of females infected after feeding on 4.8 $\log_{10}$ PFU/ml of WEEV that expectorated virus after immobilization by 3 different methods.

| Method[1] | *n* | Infected (%) | High titer[2] *n* | Trans[3] *n* |
|---|---|---|---|---|
| $CO_2$ + ice | 40 | 78 | 5 | 1 |
| Ice | 60 | 88 | 23 | 14 |
| TEA | 40 | 80 | 12 | 4 |

[1] Method of immobilization; TEA = triethylamine.
[2] Number of females with a body titer >4.7 $\log_{10}$/ml.
[3] Number of females with high titer transmitting.

= 2,131, $P = 0.006$), being greatest for the 12-day (4.7 $\log_{10}$ PFU/mosquito) and 14-day (3.8 $\log_{10}$ PFU) groups and least for the 8-day group (2.9 $\log_{10}$ PFU). Because there were no females with body titers <4.7 $\log_{10}$ PFU that transmitted virus, transmission efficiency among immobilization methods was evaluated based only on females with body titers above this threshold. There were no significant differences ($P > 0.05$) among the percentage of females transmitting WEEV when tested by a 2 × 3 chi-square (Table 4). Overall, the mean (range) titer of WEEV secreted by 19 transmitting females was 1.1 (0.5–1.7) $\log_{10}$ PFU/mosquito and did not differ significantly ($P > 0.05$) among immobilization methods. The quantity of virus secreted agreed with our previous estimates for WEEV (Reisen et al. 2000).

## DISCUSSION

Overall, colonized *Cx. tarsalis* presented with heparinized chicken blood–WEEV mixtures warmed (37°C) within a biomembrane feeder exhibited increased engorgement rates and produced WEEV infection rates comparable with or greater than mosquitoes offered blood–virus mixtures sweetened with 2.5% sucrose or a restrained chick with a natural or artificial viremia. These results were unexpected because the addition of sugar to blood–virus mixtures was anticipated to stimulate feeding by mosquitoes starved for 24 h and presumably low on glycogen reserves as well as by those females avidly seeking blood for egg production. Likewise, restrained chicks were anticipated to produce natural bird odors and $CO_2$ as added feeding stimuli. Because the feeder was warmed to 37°C, a temperature similar to that of a chick, temperature was not considered to be a differential factor. A possible difference may have been in the biomembrane made from hog intestine sausage casing. However, it was difficult to understand how a biomembrane derived from hog products filled with chicken blood would be more attractive to host-seeking females than a restrained, intact, and lightly feathered chick, especially for a species like *Cx. tarsalis* that, in nature, preferentially feeds on birds (Reisen and Reeves 1990). Another possibility involves movements by the restrained chick during feeding; however, casual observations made during our experiments indicated a greater avidity for the membrane feeder than other blood sources, including restrained chicks, even though the BFS and HVP colonies have been maintained for >20 years on chicks and/or doves as a blood meal source.

The anticoagulant sodium heparin did not appear to reduce WEEV infection rates when compared with defibrinated blood meals presented in a biomembrane feeder containing rabbit or chicken blood. Comparisons to blood meals from live chicks were variable. In experiment 2, infection rates, but not mean mosquito body titers of infected females, were somewhat higher when blood meals were taken from viremic chickens as compared with heparinized blood–virus mixtures in the biomembrane feeder. However, in experiment 3, there did not appear to be a consistent difference, with titers in females that fed on the 1-day-old chicks immediately after infection somewhat greater than females that fed on heparinized blood virus mixtures, even though the titers in the membrane feeders were greater than in the chicks. These infection results with virus presented in heparinized blood suspensions contrasted with cell culture experiments where heparin sulfate competed for binding sites and blocked infection (Bernard et al. 2000, Hurrelbrink and McMinn 2001, Su et al. 2001). Previous studies also indicated that defibrinated blood did not clot quickly in the midgut, whereas normal blood clotted rapidly and forced virus to the periphery of the blood meal adjacent to receptor sites on the midgut, thereby increasing infection (Weaver et al. 1993).

Triethylamine worked well compared with cold or $CO_2$ + cold to immobilize females for in vitro-transmission assessments using the capillary-tube method. Mosquitoes immobilized with TEA remained unable to fly and therefore could be used intact, whereas those immobilized by cold or $CO_2$ + cold had to have the wings and legs removed so that they would remain immobilized with their proboscis inserted into the capillary tube. Microscopic examination of TEA-anesthetized females revealed contractions of the alimentary tract similar to females naturally imbibing a blood meal (Reisen and Emory 1976), indicating they most likely were expectorating.

In summary, our studies indicated that heparinized blood–virus mixtures presented to colony female *Cx. tarsalis* mosquitoes in a warmed biomembrane feeder had several advantages over sweetened blood–virus mixtures presented in pledgets or as hanging drops or blood meals taken from restrained chickens with a natural or artificial viremia: 1) frequency of blood feeding was enhanced by the combination of temperature, moisture, and perhaps membrane odor; 2) infectious virus dose could be controlled; 3) infection rates were greater or similar to meals taken from viremic chickens; and 4) titers of virus in infected females were sim-

ilar to those infected by other methods. These results indicated that sodium heparin did not adversely affect the infection *Cx. tarsalis* with WEEV. The use of TEA to immobilize females for in vitro-transmission assessment using the capillary tube method produced results similar to cold or $CO_2$ + cold and provided a more efficient means of mosquito manipulation.

## ACKNOWLEDGMENTS

We especially thank Vincent Martinez and Brian Carroll, Arbovirus Field Station, and Sharon Clark and Emily Green, Arbovirus Laboratory, for excellent technical assistance. This research was funded by Research Grant 1-R01-AI47855 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Logistical support was provided by the Kern Mosquito and Vector Control District.

## REFERENCES CITED

Aitken THG. 1977. An in vitro feeding technique for artificially demonstrating virus transmission by mosquitoes. *Mosq News* 37:130–133.

Bellamy RE, Kardos EH. 1958. A strain of *Culex tarsalis* Coq. reproducing without blood meals. *Mosq News* 18: 132–134.

Bernard KA, Klimstra WB, Johnston RE. 2000. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* 276:93–103.

Cornel AJ, Jupp PG. 1989. Comparison of three methods for determining transmission rates in vector competence studies with *Culex univittatus* and West Nile and Sindbis viruses. *J Am Mosq Control Assoc* 5:70–72.

Goddard L, Roth A, Reisen WK, Scott TW. 2002. Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis* 8:1385–1391.

Hardy JL, Reeves WC. 1990. Experimental studies on infection in vectors. In: Reeves WC, ed. *Epidemiology and control of mosquito-borne arboviruses in California, 1943–1987* Sacramento, CA: California Mosquito Vector Control Association. p 145–250.

Hintze JL. 1998. NCSS statistical software (computer program). Kaysville, UT: NCSS.

Hurrelbrink RJ, McMinn PC. 2001. Attenuation of Murray Valley encephalitis virus by site-directed mutagenesis of the hinge and putative receptor-binding regions of the envelope protein. *J Virol* 75:7692–7702.

Kramer LD, Bowen MD, Hardy JL, Reeves WC, Presser SB, Eldridge BF. 1993. Vector competence of alpine, Central Valley, and coastal mosquitoes (Diptera: Culicidae) from California for Jamestown Canyon virus. *J Med Entomol* 30:398–406.

Kramer LD, Presser SB, Houk EJ, Hardy JL. 1990. Effect of the anesthetizing agent triethylamine on western equine encephalomyelitis and St. Louis encephalitis viral titers in mosquitoes (Diptera: Culicidae). *J Med Entomol* 27:1008–1010.

Lee E, Lobigs M. 2002. Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. *J Virol* 76:4901–4911.

Mahmood F. 1999. Laboratory maintenance of mosquitoes. In: Maramorosch K, Mahmood F, eds. *Maintenance of human, animal and plant pathogen vectors* Enfield, NH: Scientific Publishers Inc. p 3–31.

O'Guinn M, Turell MJ. 2002. Effect of triethylamine on the recovery of selected South American Alphaviruses, Flaviviruses, and Bunyaviruses from mosquito (Diptera: Culicidae) pools. *J Med Entomol* 39:806–808.

Reisen WK, Chiles RE, Kramer LD, Martinez VM, Eldridge BF. 2000. Method of infection does not alter the response of chicks and house finches to western equine encephalomyelitis and St. Louis encephalitis viruses. *J Med Entomol* 37:250–258.

Reisen WK, Emory RW. 1976. Blood feeding of *Anopheles stephensi*. *Ann Entomol Soc Am* 69:293–298.

Reisen WK, Hardy JL, Presser SB. 1997. Effects of water quality on the vector competence of *Culex tarsalis* (Diptera: Culicidae) for western equine encephalomyelitis (Togaviridae) and St. Louis encephalitis (Flaviviridae) viruses. *J Med Entomol* 34:631–643.

Reisen WK, Meyer RP, Presser SB, Hardy JL. 1993. Effect of temperature on the transmission of western equine encephalomyelitis and St. Louis encephalitis viruses by *Culex tarsalis* (Diptera: Culicidae). *J Med Entomol* 30:151–160.

Reisen WK, Reeves WC. 1990. Bionomics and ecology of *Culex tarsalis* and other potential mosquito vector species. In: Reeves WC, ed. *Epidemiology and control of mosquito-borne arboviruses in California, 1943–1987* Sacramento, CA: California Mosquito and Vector Control Association. p 254–329.

Rutledge LC, Ward RA, Gould DJ. 1964. Studies on the feeding response of mosquitoes to nutritive solutions in a membrane feeder. *Mosq News* 24:407–419.

Su CM, Liao CL, Lee YL, Lin YL. 2001. Highly sulfated forms of heparin sulfate are involved in Japanese encephalitis virus infection. *Virology* 286:206–215.

Turell MJ. 1988. Reduced Rift Valley fever virus infection rates in mosquitoes associated with pledget feedings. *Am J Trop Med Hyg* 39:597–602.

Weaver SC, Lorenz LH, Scott TW. 1993. Distribution of western equine encephalomyelitis virus in the alimentary tract of *Culex tarsalis* (Diptera: Culicidae) following natural and artificial blood meals. *J Med Entomol* 30:391–397.