Limited Interdecadal Variation in Mosquito (Diptera: Culicidae) and Avian Host Competence for Western Equine Encephalomyelitis Virus (Togaviridae: Alphavirus)

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Abstract. Historically, western equine encephalomyelitis virus (WEEV) caused large equine and human epidemics in the Americas from Canada into Argentina. Despite recent enhanced surveillance for West Nile virus, there have been few reports of equine or human cases and little documented enzootic activity of WEEV. During the past three years, WEEV has been active again in California, but without human or equine cases. In the current study, we compared host and vector competence of representative WEEV isolates made during each decade over the past 60 years using white-crowned sparrows, house sparrows, and Culex tarsalis Coquillett as representative hosts. Results indicated limited time-related change in virulence among WEEV strains in birds and little difference in vector competence in Cx. tarsalis. Although temporal and spatial genetic changes have been documented, these seem to present limited phenotypic change in host competence and cannot explain the absence of equine and human cases.

INTRODUCTION

Historically, western equine encephalomyelitis virus (WEEV, Togaviridae, Alphavirus) caused devastating public and veterinary health problems in the western United States. This was especially true in the Central Valley of California, where equine epizootics crippled agriculture at a time when mules and horses were the primary force that powered farm equipment. In addition, outbreaks of human illness were common, especially in young children, with many adults having acquired immunity. The advent of widespread immunization of equines and ratite birds, completion of water management projects along the western slope of the Sierra Nevada, increased use of air conditioning and window screening, reduced environmental exposure of humans, and expanded mosquito control programs have combined to almost eliminate WEEV as a human and equine health problem in California and the remainder of western North America. Despite the dramatic decrease in epidemic/epizootic activity, widespread enzootic activity was documented in California during 1978–1979, 1983–1984, 1993, and most recently 2005–2007. Interestingly, these increases in enzootic transmission were not accompanied by tangential transmission to humans and equines as shown by the California human population being essentially immunologically naïve. In contrast, WEEV remains a veterinary problem in Argentina where equine cases continue to be reported.

The cause for the decrease in human WEEV infection remains poorly understood. A comparison of the partial sequences of strains of WEEV isolated in California since 1938 delineated four lineages consisting of isolates from 1) Central Valley and Los Angeles, 1993–1998, 2) Southern California after 1978 and the Central Valley from 1978 through 1987, 3) Northern California from 1968 through 1971, and 4) the oldest Californian strains isolated between 1938 and 1961. Avian host competence studies using WEEV strains isolated during 1953–1971 indicated considerable virulence among recipient passeriform birds, especially white-crowned sparrows (Zonotrichia leucophrys) that had mortality rates ranging from 30% to 85%. Despite an absence of human cases, several isolates associated with the 1993 epizootic in the Sacramento Valley had high neurovirulence and neuroinvasiveness in a suckling/weanling mouse model. All of these strains replicated to high titers in one-day-old chickens. In contrast, host competence experiments conducted with WEEV strains isolated in California between 1983 and 1998 indicated markedly less virulence in birds than the BFS1703 strain isolated in 1953. A direct comparison of 1953 and 1983 isolates in WEEV demonstrated a significant decrease in viremia and mortality for the 1983 isolate. Collectively, these data indicated that the decrease in WEEV equine and human cases may be linked to an attenuation of the virus that limited amplification within the avian enzootic cycle and therefore subsequent tangential transmission to mammals. The absence of WEEV infection in Aedes melani-mon Meigen since 1983 may be indicative of low enzootic amplification and spill-over into the secondary rabbit-Aedes cycle.

After being absent from the Central Valley since 1998, WEEV reappeared in the southeastern California deserts during 2002 and then in Kern County during 2005 where it has remained active during each of the following summers without reported human cases, prompting us to initiate studies on the genetics of these recent isolates (Brault AC, unpublished data). During 1999–2001, WEEV was not detected despite the testing of 10,968 mosquito pools and sera from 130–190 flocks of sentinel chickens. To assess the hypothesis that reduced vector competence and/or replication in avian maintenance hosts restricts the potential for epidemic/epizootics, we compared the vector and host competence of an isolate obtained from the Imperial Valley in 2005 with isolates obtained historically between 1953 and 1992. Concurrent or sequential dual infections may increase the virulence of a pathogen in a host that normally is not competent. For example, concurrent infection of domestic pigeons (Columba livia) with Newcastle and West Nile viruses (WNVs) resulted in mortality and increased viremias caused by WNV. Therefore, to determine if previous infection with WNV affects the WEEV viremia response, we compared WEEV viremia profiles in birds with and without naturally occurring antibody to WNV.
MATERIALS AND METHODS

Viruses. We used strains of WEEV isolated from pools of *Culex tarsalis* during each decade from 1950 through 2005 (Tables 1 and 2). All strains were isolated from the Bakersfield area of Kern County except for BFN3060, which was isolated from Butte County near Chico; COA592, which was isolated from Coachella Valley near the Salton Sea in Riverside County; and IMP181, which was isolated near the Salton Sea in Imperial County. Prior to 1990 isolations were made by sucking mouse intracerebral inoculation, whereas after 1990 all isolates made in Vero cell culture. All strains were at sucking mouse or Vero cell passage 2 at the time of experimentation. Strains BFS1703, KERN5547, and COAV592 were used in previous avian host and vector competence experiments and have been partially sequenced.

Mosquitoes. Two recently established *Cx. tarsalis* Coquillett colonies were used because this species is the primary enzootic and epidemic vector of WEEV in California. The Kern National Wildlife Refuge (KNWR) colony was established from host-seeking females collected at the Refuge in northern Kern County in 2003. The Yolo County colony was established from host-seeking females collected from the Yolo by-pass of the Sacramento River in Yolo County of northern California in 2005. All mosquitoes were reared at a standard density of four egg rafts per enamel tray at temperatures of 22–24°C and photoperiod of 16:8 hours light:dark. A total of 60–80 females that were within 3–8 days post-emergence were sorted into 0.6-liter infection cartons and starved for 24 hours prior to infection attempts.

Birds. The WCSPs and house sparrows (HOSPs, *Passer domesticus*) were selected as experimental hosts in which to compare WEEV strains. The WCSPs were highly susceptible to WEEV in previous studies and showed marked differences between BFS1703 and KERN5547 viral strains in one preliminary experiment. In contrast, HOSPs were previously shown to be a moderately competent host for recent strains of WEEV. Western scrub jays (WESJs, *Aphelocoma californica*) and house finches (HOFIs, *Carpodacus mexicanus*) collected at the Kern River near Bakersfield were used to determine the effects of previous WNV infection on the viremia response because both species frequently were found to be WNV seropositive in the field. All birds were collected at grain-baited traps operated near our laboratory in Bakersfield, Kern County, California and were maintained on mixed wild bird seed. Birds were prebled to determine previous exposure, housed 4–6 per cage (except WESJs, which were housed individually) within our mosquito-proof containment facility, and allowed 1–2 weeks to acclimate to cage conditions prior to experimental inoculation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Virus</th>
<th>Titer*</th>
<th>n</th>
<th>No. infected (%)</th>
<th>No. transmitted (%)</th>
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</thead>
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<tr>
<td>KNWR</td>
<td>BFS1703</td>
<td>6.4</td>
<td>25</td>
<td>25 (100)</td>
<td>15 (60)</td>
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<tr>
<td>IMP181</td>
<td>BFS1703</td>
<td>5.9</td>
<td>25</td>
<td>24 (96)</td>
<td>12 (50)</td>
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<tr>
<td>YOLO</td>
<td>IMP181</td>
<td>5.7</td>
<td>17</td>
<td>14 (82)</td>
<td>6 (43)</td>
</tr>
<tr>
<td></td>
<td>IMP181</td>
<td>5.7</td>
<td>17</td>
<td>5 (71)</td>
<td>3 (60)</td>
</tr>
</tbody>
</table>

* Infected dose in log10 plaque-forming units/mL.
† % = Transmitting/infected x 100.

Ethics. The collection and infection of wild birds with encephalitis viruses was done under Protocol 11184 approved by the Institutional Animal Care and Use Committee of the University of California, Davis, California Resident Scientific Collection Permit 801049-02 by the State of California Department of Fish and Game, and Federal Fish and Wildlife Permit No. MB082812-0. Use of arboviruses was approved under Biological Use Authorization #0554 by Environmental Health and Safety of the University of California, Davis, and U.S. Department of Agriculture Permit #47901.

Experiments. Viruses were compared in the following experiments.

**Experiment 1: Vector competence.** *Culex tarsalis* from the Yolo and KNWR colonies were offered a 10-fold dilution series of BFS1703 or IMP181 viruses in heparinized chicken blood containing 2.5% sucrose. Yolo females were fed on cotton pledges soaked with the virus mixture, and KNWR females were fed on a similar solution presented through a biomembrane on a Hemotek apparatus (Discovery Works, Accrington, United Kingdom). Females were allowed to feed for up to 1 hour, after which engorged females were transferred to clean 0.6-liter cartons and maintained for 14 days in an incubator at 26°C. After the 14-day extrinsic incubation period, females exposed to the highest titer of virus were anesthetized with triethylene and their proboscis inserted into a capillary tube filled with 0.1 mL of 50% fetal bovine sera (FBS) and 10% sucrose in distilled water. After 10–15 minutes, the contents of the capillary tube were expelled into 0.3 mL of diluent (buffered saline with 15% FBS and antibiotics). Expectorate samples and bodies of these females as well as those exposed to lower concentrations of WEEV were placed in individual cryovials and stored at –80°C until tested. Transmission rates were calculated as the percentage of females with positive expectorate samples divided by the number of infected females tested.

**Experiment 2: Host competence.** Groups of 4–8 WCSPs or HOSPs were inoculated subcutaneously in the cervical region with 2–3 log10 plaque-forming units (PFU)/0.1 mL of each WEEV strain. To monitor viremia, birds were bled daily by jugular puncture with 0.1 mL of blood collected using...
28-gauge needles, expressed into 0.4 mL of virus diluent, centrifuged, and frozen immediately at −80°C until assessed for viral titer by titration by plaque assay on Vero cells.

Experiment 3: Effect of antibody to WNV on WEEV infection. This experiment tested the notion that birds previously infected with WNV may have a different response to WEEV infection than those not previously infected, despite the fact that these viruses are in different families and are antigenically distinct. Four HOFIs and three WESJs that were antibody positive by enzyme immunoassay (EIA) for WNV and an equal number of birds that were antibody negative each were inoculated subcutaneously in the cervical region with a 2.7 log_{10} PFU/0.1 mL of inoculum of the KERN5547 strain of WEEV. Birds were bled daily for five days to assess viremia and then weekly for six weeks to assess the development of antibody.

Assays. Mosquito bodies were triturated in 1 mL of diluent, and RNA was extracted using an ABI 6100 (Applied Biosystems, Foster City, CA) workstation and tested using a real-time reverse transcription–polymerase chain reaction with an ABI 7900 TaqMan work station using primers developed for strains of WEEV isolated from California.26 Mosquito expe- torate and bird viremia samples were tested for WEEV using a standard plaque assay on Vero cells.27 Antibody samples (including prebleeds) were tested using an EIA with WEEV or St. Louis encephalitis virus (SLEV) antigen, with antibody levels expressed as the positive/negative ratio of the mean optical density of two antigen positive wells (P) divided by the optical density for one antigen negative well (N) per antibody sample.28 Neutralizing antibody titer was measured by standard 80% plaque-reduction neutralization assay (PRNT_{80}) on week six post-infection.29

RESULTS

Experiment 1: Vector competence. The infection-dose response curves for the KNWR colony of Cx. tarsalis ingesting a 10-fold dilution series of BFS1703 or IMP181 strains of WEEV were almost identical as were the median infectious doses (ID_{50}) estimated by probit analyses (Figure 1). When assessed using the in vitro capillary tube method,25 there were no significant differences (χ² < 1.6, degrees of freedom [df] = 1, P > 0.2) in the ability of either the KNWR or YOLO strains of Cx. tarsalis to transmit the BFS1703 and IMP181 strains of WEEV (Table 1). The mean ± SD of BFS1703 virus expec- torated by Cx. tarsalis (1.36 ± 0.42 log_{10} PFU) was not signifi- cantly different (t = 0.86, df = 29, P = 0.2) than that of IMP181 virus (1.23 ± 0.41 log_{10} PFU). Collectively, these data indicated that there were minimal differences in the vector competence of Cx. tarsalis for the 1953 and 2005 WEEV strains.

Experiment 2: Host competence. All birds were negative by EIA at capture for antibodies against WEEV and SLEV. The response of WCSPs and HOSPs to infection with representative strains of WEEV were relatively similar, with viremias peaking on days 1 or 2 post-infection and then rapidly decreasing to < 1.7 log_{10} PFU/mL (the minimal detection threshold of our plaque assay) by days 3 to 5 (Figure 2). Significantly more birds, two of eight WCSPs and one of eight HOSPs, infected with the 1953 BFS1703 strain died during acute infection (χ² = 11.1, P < 0.001) than did the remaining 57 infected birds that survived after infection with the remain- ing WEEV strains (Table 2). Four uninfected procedural control WCSPs also survived the handling and repeated sampling. When tested using a repeated measures analysis of variance,30 there were significant differences in mean viremias on 1 and 2 days post-infection among WEEV strains within bird species (WCSPs: F = 6.23, df = 5, 40, P < 0.001; HOSPs: F = 12.79, df = 3, 23, P < 0.001). For both bird species, the COA592 strain isolated in 1992 and the BFS1703 strain iso- lated in 1953 had the highest mean viremias, whereas the IMP181, KERN5547, and BFS4740 strains had the lowest mean viremias (Figure 2 and Table 2).

Experiment 3: WNV immunity. All birds were negative by EIA at capture for antibodies against WEEV. As expected, previous infection with WNV had little effect on the course of WEEV infection in WESJs and HOFIs with the KERN5547 strain. WEEV and WNV are in different viral families, do not cross react serologically, and do not provide cross-protective immunity. The WESJs and HOFIs with naturally acquired immunity to WNV did not have a different viremia response to infection with the KERN5547 strain of WEEV than did birds without WNV antibody (Figure 3). Although we ini- tially planned to concurrently compare three WESJs with and without WNV antibody, some of the birds negative for WNV by EIA at capture later developed antibodies to WNV before experimentation and were positive for antibodies when tested against WNV at one week post-infection (Table 3, bird 1286 was negative at week 1, but positive on other bleed). Therefore, these WNV antibody–positive WESJs were included as positive samples in Figure 3. For comparison, the mean vire- mia from five negative WESJs infected with KERN5547 in May 2000 before WNV arrived in California27 were included in Figure 3 as ‘WNV neg’ for comparison. The mean ± SD viremia for these birds on day 1 (2.5 ± 1.0 log_{10} PFU/mL) was not different (t = −0.4, df = 8, P = 0.35) from positive birds in the current study (2.4 ± 0.4 log_{10} PFU/mL). Similarly there was no statistical difference (P > 0.05) in WEEV viremias for HOFIs bled on day 1 that were positive and negative for antibody to WNV (Figure 3 and Table 3). Overall, there also were no significant differences on day 1 post-infection be- tween HOFIs (2.5 ± 0.3 log_{10} PFU/mL) and WESJs (2.4 ± 0.4 log_{10} PFU/mL). However, the overall day 2 post-infection HOFIs mean viremia (± 95% confidence limit) was 3.1 (1.2)
log_{10} PFU/mL and was significantly greater than that of the WESJs that were negative (i.e., < 1.7 log_{10} PFU/mL). The PRNT_{so} antibody against WNV in week 6 was < 1:20 for negative birds, but ≥ 1:40 for positive birds. Two exceptions were WESJ1285 and HOFI 56 that had negative EIA positive/negative ratios but low PRNT_{so} titers. The PRNT_{so} titers against WEEV were statistically similar to those for HOFIs that were (mean = 1:160) and were not (mean = 1:226) previously infected with WNV (log_{10} transformed, t = 0.8, df = 6, P = 0.5), which indicated that previous infection with WNV did not alter the immune response to WEEV.

DISCUSSION
There did not appear to be temporal patterns in vector or host competence that would account for decreases in WEEV enzootic amplification and the reduction of equine and human cases. There were essentially no differences in *Cx. tarsalis* vector competence for the 1953 BFS1703 and the 2005 IMP181 strains of WEEV. In contrast to vector competence, there were significant differences among the avian responses to infection with WEEV isolates, but these did not assort by time. The BFS1703 strain isolated in 1953 from Kern County exhibited among the highest viremias and was the only strain that produced mortality in birds, which was consistent with results of previous studies. The COA592 strain isolated from Coachella Valley in 1992 produced statistically similar viremia titers, but without mortality. The BFS1703 strain was isolated after the large 1952 epidemic, whereas the COA592 strain was isolated during increased enzootic activity in Coachella Valley in 1992. The greatest viremia decrease was seen in the response of HOSPs to the IMP181 strain (mean titer = 3.6 log_{10} PFU/mL). Three additional strains isolated from 1964 through 2005 exhibited low mean titers in WCSPs, ranging from 4.8 to 5.5 log_{10} PFU/mL. Further research will be necessary to elucidate factors leading to variation in virulence among avian but not mosquito hosts. There were differences in the history of exposure of these two bird species to
WEEV. The WSCP subspecies gambelli found in Kern County spends summers in Alaska\textsuperscript{32} and therefore is absent during most of the transmission season. In contrast, although HOSPs originated from Europe and did not arrive in the Central Valley until approximately 100 years ago, these populations have been exposed to WEEV during most summers since that time.

Peak viremias and percent mortality data in the current study were markedly lower than those observed in studies done previously using the BFS1703 and other WEEV strains isolated from 1957 through 1962.\textsuperscript{14} In these studies, WCSPs and HOSPs infected with the BFS1703 strain attained mean peak viremias measured by plaque assay of $> 10$ and $> 9 \log_{10}$ PFU/mL at 24 hours post-infection, respectively, which are three and two orders of magnitude greater than that observed in the current study. Although birds in these previous studies were maintained in outdoor cages, bled with 26-gauge needles, and 0.2-mL blood samples were obtained every second day, these husbandry differences most likely could not resolve these disparities in viremias. In addition, all viral strains in our experiments and previous experiments were isolated from \textit{Cx. tarsalis}, stored at $-80^\circ$C, and amplified in Vero cells prior to study. Thus, virus isolation procedures probably had minimal impact on viremia results. Therefore, the only likely source of these differences was the susceptibility status of the WCSPs and HOSPs used for experimentation. Unless avian gene markers for these changes in susceptibility can be found, there is little hope of resolving these apparent differences in host competence.

Comparison of WEEV and WNV infections in the same hosts showed greater vector susceptibility (i.e., lower ID\textsubscript{50}) and lower avian viremia for endemic WEEV compared with the invading WNV. \textit{Culex tarsalis} from Kern County were much more susceptible to WEEV, with an estimated ID\textsubscript{50} of 3.2–3.4 $\log_{10}$ PFU/mL than the NY99 strain of WNV, which had an ID\textsubscript{50} of 4.4 $\log_{10}$ PFU/mL.\textsuperscript{33} Ranges of mean peak avian viremias were generally lower for WEEV, although overlapping with WNV: HOSPs peak viremia range = 3.8–6.9 $\log_{10}$ PFU/mL for WEEV versus 6.0 for WNV; WCSPs = 4.8–6.5 $\log_{10}$ PFU/mL for WEEV and 6.8–7.5 $\log_{10}$ PFU/mL for WNV. In addition, WEEV replicates better in \textit{Cx. tarsalis} under cool temperatures than WNV and therefore potentially has a longer transmission season than WNV.\textsuperscript{34} With a lower mosquito ID\textsubscript{50} and lower thermal threshold for replication, \textit{Cx. tarsalis} would be expected to be more readily infected and transmit more efficiently at the avian viremia titers produced by WEEV than at the slightly higher titers for WNV. However, this has not been the case and, although WNV has continued to be transmitted at epidemic levels in California, WEEV has remained at low enzootic levels without reported human cases during the past few years.\textsuperscript{35,36} Apparently, the decrease in human cases must be related to epidemiologic factors such as improved housing and the use of air conditioning, whereas the decrease in equine cases most likely is related to extensive vaccination.

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REFERENCES


