High Subclinical West Nile Virus Incidence among Nonvaccinated Horses in Northern California Associated with Low Vector Abundance and Infection

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Abstract. Although horse cases frequently are reported during West Nile virus (WNV) outbreaks, few investigations have focused on the epidemiology of this transmission. From April to October 2003 to 2005, mosquito abundance and infection were monitored 3 days per week at an equine research facility at the University of California, Davis. Thirty-two nonvaccinated horses enrolled as controls in a vaccine study were bled monthly, and their serum was tested for evidence of WNV infection by plaque reduction neutralization test (PRNT). In 2004, one positive Culex pipiens pool was associated with a single horse that presented with confirmed WNV disease in late September. The annual incidence of clinical and subclinical WNV infection in the nonvaccinated horses was 16%, with an apparent to inapparent ratio of 1:4 among infected horses. In 2005, two Culex tarsalis and two Cx. pipiens WNV-positive pools were associated with an equine infection incidence of 62%, with an apparent to inapparent ratio of 1:17. The majority (79%) of 70 blood-engorged Cx. pipiens fed on birds and the remaining on equines (21%). Conversely, Cx. tarsalis fed primarily on equines (n = 23, 74%), followed by birds (n = 7, 23%) and 1 (3%) fed on a lagomorph. These data indicated that nonvaccinated horses were a sensitive indicator of WNV activity and that their risk of infection was associated with the presence of infection in Cx. pipiens and Cx. tarsalis, which served as both enzootic and bridge vectors amplifying WNV among birds and transmitting WNV to horses.

INTRODUCTION

The West Nile virus (WNV) epizootic in the U.S. has been severe, with > 24,600 equine cases reported by the end of 2006.1 Case-fatality rates of clinically afflicted equines are estimated to be as high as 35–42%.2–4 Once infected with WNV, nonimmune horses are susceptible to clinical neuroinvasive disease manifested as ataxia (stumbling, staggering, wobbly gait, or incoordination), muscle fasciculation, inability to stand, multiple limb paralysis, or death.5 However, similar to humans, most equine infections are subclinical or inapparent, and although experimental infection studies indicate that ca. 10% of infected horses develop clinical illness,5 the apparent to inapparent ratio from prospective epidemiologic studies remains unknown.

Arbovirus surveillance programs were established or restructured in 48 states after the emergence of WNV in North America and have included monitoring equine cases and testing mosquito pools to detect the presence of the virus and virus transmission activity. The utility of using equine cases in WNV surveillance is variable, often disputed and can be confounded by vaccination efforts that have been in place since 2003.6,7 The USDA, APHIS Veterinary Services, and California Department of Food and Agriculture (CDFA) classify a confirmed case of equine WNV infection as having compatible clinical signs and supplementary laboratory findings, such as isolation of WNV from tissue; positive reverse-transcriptase polymerase chain reaction (RT-PCR) for WNV genomic sequences in tissues; detection of IgM antibody to WNV by IgM-capture enzyme linked immunosorbent assay (ELISA) in serum or cerebral spinal fluid (CSF); and/or a 4-fold or greater increase in plaque-reduction neutralization (PRNT) antibody titer to WNV in appropriately timed, paired sera for nonvaccinated horses.2,3,8 An exposed or subclinically infected equine exhibits IgM antibody to WNV detected by IgM-capture ELISA in serum and/or CSF or a 4-fold rise in IgG antibody, without any observable clinical signs.8

The introduction of WNV into New York state in 1999 was followed by a rapid westward expansion across the United States. Above-average summer temperatures have been linked with this rapid dispersal, especially at northern latitudes.9 WNV eventually invaded Southern California in 200310 and dispersed to Northern California in 2004.11 In 2005, Sacramento County was the epicenter of WNV activity for all of California, with a marked increase of reported cases in human, horse, and avian populations.12 Although the true equine population in California is unknown, in 2005 it was estimated to be approximately 698,000 horses by the American Horse Council13 and is considered an important component of California's agricultural economy. During 2004, 540 equine cases were confirmed in California, of which 230 died or were euthanized with a resulting case-fatality rate of 43%.4 In 2005, the CDFA reported 456 equine cases with 200 deaths, a case-fatality rate of 44%.14 Several studies have focused on the role of different mosquito species in bird to bird and bird to human WNV transmission in North America.5 The species of mosquitoes transmitting WNV to horses are not as well understood. Due to the large amounts of morbidity and mortality from WNV infection in horses, it is essential to identify which mosquito species are risk factors for equine infection. Historically in California, Cx. tarsalis was involved in extensive western equine encephalomyelitis virus (WEEV) equine outbreaks, whereas members of the Cx. pipiens complex are not competent vectors of WEEV15 and therefore were not involved in these early equine epizootics. However, Cx. pipiens are considered important in St. Louis encephalitis virus (SLEV) and WNV avian-vector amplification cycles because of their abundance, ornithopagous feeding patterns, and susceptibility to infection.16,17 Although its role has varied regionally, this species is considered to be an important enzootic vector throughout the United States.18–20 For example, eastern U.S. Cx. p. pipiens
populations appear to feed predominantly on birds.\textsuperscript{21} In contrast, \textit{Cx. pipiens} in the Sacramento Valley were previously shown to be introgressed with \textit{Culex quinquefasciatus} genes\textsuperscript{22} and feed on both birds and mammals, including horses.\textsuperscript{23}

Opportunist mosquito species such as \textit{Cx. tarsalis} feed on both birds and mammals, with a shift to more mammalophagic feeding during late summer\textsuperscript{24} and therefore may function as maintenance, amplification, and bridge vectors to incidental hosts such as humans and horses.\textsuperscript{25} When WNV spread to the southeast and Great Plains states in 2001 and 2002, an exponential increase in equine cases occurred.\textsuperscript{7} This exponential increase likely reflects the spread of WNV into regions with competent vectors, such as \textit{Cx. quinquefasciatus} and \textit{Cx. tarsalis}, that frequently feed on horses.\textsuperscript{7} Additionally, 10 mosquito species from California were found experimentally to be competent vectors of WNV.\textsuperscript{18} Although competence varied widely, \textit{Cx. tarsalis} from Yolo County was the most competent population of those tested.\textsuperscript{18}

The primary goal of the current study was to prospectively relate the temporal association of mosquito abundance, host-feeding patterns, and WNV infection rates in field-collected mosquitoes to infection rates in a cohort of nonvaccinated and previously unexposed horses. By comparing the temporal changes in mosquito populations and vector infection rates to the occurrence of equine infections, we were better able to delineate entomological risk factors that are required for tangential transmission to horses.

**MATERIALS AND METHODS**

**Equine serology.** Thirty-two nonvaccinated horses that previously were not exposed to WNV were selected randomly as controls in a vaccine study that commenced in 2003 and continued until November 2005.\textsuperscript{26} These nonvaccinated control horses were maintained at the Center for Equine Health (CEH), a 24-hectare equine facility in Davis, California (38°31’ N, 121°45’ W), with over 150 vaccinated horses, from the time of enrollment until the end of the study or until attrition occurred. The monitored cohort was housed in corrals and open pastures and freely intermingled with horses that were vaccinated against WNV in groups of 6–10 horses. All of the horses were monitored daily for evidence of clinically apparent WNV infection or any other illness by trained animal-health technicians. Horses that presented with clinical signs were evaluated by veterinarians, and WNV infection was confirmed by IgM-capture ELISA, which was performed at the California Animal Health and Food Safety (CAHFS) Laboratory, and by the plaque-reduction neutralization test (PRNT), performed at the National Veterinary Services Laboratory (NVSL). In addition, blood was collected monthly from all horses, and the serum was separated and stored at –20°C until it was tested. The protocol for the care, maintenance, and collection of blood samples from these horses was approved by the University of California Institutional Animal Care and Use Committee.

Samples from naturally or experimentally infected horses have shown that IgM antibodies are first detectable 8–10 days post-infection and begin to decay approximately 2–3 months after infection.\textsuperscript{5,27} Although WNV-neutralizing (IgG) antibodies are not detectable until 2 or more weeks after infection, they can persist for more than 2 years.\textsuperscript{27} Therefore, sera were tested for the presence of neutralizing antibodies to WNV by PRNT at the Center for Vectorborne Diseases (CVEC) arbovirus laboratory. A 90% reduction of 100 plaque-forming units (PFU) of the NY99 strain of WNV (2nd Vero cell passage) at a dilution of $\geq 1:20$ was considered presumptive evidence of infection. Horses with serologic results that were indicative of previous exposure to WNV (i.e., horses that had WNV-positive titers from sera collected at the beginning of the study) or horses that were not present for the entire study period each year were excluded. A 2-tiered screening process was used to minimize testing. Sera collected in March 2004 from the nonvaccinated horse cohort before any evidence of WNV was detected at the CEH were tested to confirm that previous exposure to WNV had not occurred. Sera collected in December of 2004 from a total of 32 confirmed unexposed horses were tested by PRNT. Horses with WNV-positive titers in December had sera collected from April to November 2004 tested to determine the month when seroconversion occurred. Therefore, a negative PRNT test from sera collected March 2004 along with $\geq 1:20$ PRNT titers from a sequential sample, of which the previous month was PRNT negative, was considered positive evidence of a seroconversion and recent infection. Sera collected in 2005 from the remaining unexposed horses were screened in the same manner, except the last sera were collected in November when the study was terminated.

**Mosquito sampling and processing.** From April to October, from 2003 to 2005, adult mosquitoes were collected at 6 fixed sites, approximately 0.3 km apart, throughout the CEH. At each site, one dry-ice–baited trap\textsuperscript{26,29} and one gravid trap\textsuperscript{30} were set 3 consecutive nights per week, weather permitting. The traps were located at vegetative ecotones near open pastures, barns, and holding pens. After each trap night, mosquitoes were anesthetized with triethylamine, identified, and enumerated by species. Female \textit{Culex} were grouped into pools containing 10–50 individuals per trap type from all trap sites throughout the equine center; daily mosquito collections of less than 10 per species were not tested due to budget constraints. The mosquito pools were then tested for the presence of WNV, SLEV, and WEEV viral RNA by real-time, multiplex RT-PCR at the CVEC arbovirus laboratory at the University of California, Davis.\textsuperscript{31} Daily minimum and maximum temperatures from 2004 and 2005 were downloaded from the California Integrated Pest Management project website (http://www.ipm.ucdavis.edu, accessed November 15, 2006), from a weather station located 2.5 km northwest from the CEH grounds (38°32’ N, 121°47’ W), to relate WNV activity and transmission with monthly average temperatures.

**Blood-meal identification.** Three walk-in red boxes (1.8 m x 1.2 m x 1.2 m)\textsuperscript{32} were set on the CEH grounds, at approximately 0.4 km apart, and sampled from June to October, in 2004 and 2005, to collect blood-engorged resting female mosquitoes. Three mornings per week mosquitoes were removed from the walls of the boxes by handheld aspirator. Female mosquitoes were anesthetized, identified to species, scored according to level of blood engorgement, and stored separately at –80°C until they were tested to determine blood-meal host. Individual mosquitoes then were thawed and ground by adding 150 µL of PBS and EDTA and one copper-coated BB to each vial and vortexing by hand until the abdominal contents were sufficiently mixed. The color of the supernatant was recorded for each specimen from 1 (clear) to 5 (bright red), and only samples scoring $\geq 2$ were tested. To
kill any virus present, each mosquito titrate was heat inactivated at 56°C for 20 minutes. A modified sandwich ELISA was used to detect human, equine, bovine, lagomorph, and avian IgG. The avian reagent was a polyclonal anti-bird antibody able to detect all avian species within our study area. Control mosquitoes that imbibed blood from known vertebrate species were used to confirm the specificity of the reagents and to determine the optical density (OD) cutoffs for positive identification. Five groups of 25 laboratory-reared Cx. pipiens that had fed on blood from a horse, cow, chicken, rabbit, or human were included as controls. Positive cutoff values were determined to be 3 standard deviations above the mean OD readings from the negative control wells. Positive field-collected mosquitoes were retested for the host for which a positive reading had occurred. If the second ELISA results agreed with the initial test, the sample was considered to be confirmed positive. Monthly avian-to-equine blood-meal ratios were compared for both Cx. pipiens and Cx. tarsalis to determine if seasonal differences could be detected.

Statistical analyses. Data were analyzed using Minitab version 15 (Minitab Inc., State College, PA) and MedCalc version 9.2.1.0 (MedCalc Software, Mariakerke, Belgium). Descriptive statistical analyses were conducted on the 2004 and 2005 temperature data, the demographic horse data, and the abundance of Culex mosquitoes collected by trap type, species, and year. All variables were checked for normality by the Kolmogorov–Smirnov test. Multiple comparisons for the difference of means were performed on normally distributed Culex trap collection data, 2003–2005, using Tukey’s highest significant difference adjustment for comparisons of means. A nonparametric Kruskal–Wallis test for $K = 3$ was performed on the data that were not normally distributed to determine if the sample means were equal between the 3 years. Minimum infection rates (MIR) in the mosquitoes were calculated to quantify the minimum number of infected individuals per 1,000 mosquitoes using a maximum likelihood estimate (MLE).

Incidence density rates of WNV clinical and subclinical equine infections were determined for 2004 and 2005 using the cumulative horse-months per year that each horse contributed while enrolled in the study for the denominator. Poisson rates were used to detect differences in the seroprevalences between 2004 and 2005 to adjust for the differences in total amount of time each horse contributed to the study. A survival analysis was conducted on the equine serology results, and Kaplan–Meier survival curves were plotted, using the observed month that seroconversion occurred for each positive horse in 2004 and 2005. A hazard ratio was calculated to compare the survival curves for each year to determine if the relative risk of WNV infection was greater for 1 year.

RESULTS

Equine serology. WNV was detected in mosquito pools before clinical illness occurred in the individual horses that were affected during both 2004 and 2005. Serological results indicated, however, that horses were subclinically infected in 2004 before virus was detected in mosquitoes (Figure 1). Serum collected March 20, 2004 from 32 nonvaccinated horses was negative for antibody against WNV by PRNT. One of the study horses presented with clinical illness and was confirmed to be infected with WNV in late September 2004 and was euthanized on October 2. Serum collected from this horse on September 29 was PRNT-positive (90% plaque reduction, > 1:80). Of the remaining nonvaccinated cohort, 4 additional horses seroconverted (90% plaque reduction, > 1:80) by the end of the year but did not present clinical manifestations of WNV illness. PRNT results indicated 1 horse seroconverted by July 28 and a second horse seroconverted by August 17. The 2 remaining horses with subclinical infections seroconverted by September 29. These results indicated an annual incidence of 16% for equine WNV infection. Positive horses were removed from the study. One additional horse was lost from an unrelated death (colic).

In 2005, the nonvaccinated and unexposed cohort consisted of 29 horses, including 3 newly recruited horses. Serum collected January 29, 2005, was PRNT-negative for all horses. The 2005 annual incidence of 62% was significantly greater than 16% in 2004 ($P < 0.001$). One horse presented with clinical illness on August 8, was positive for WNV infection by ELISA conducted at the CDFA, and later recovered. An additional 17 subclinical infections were detected by seroconversion; 12 occurred in July, 1 in August, 3 in September, and 1 in October. Figure 1 illustrates the monthly number of equine WNV infections for 2004 and 2005 compared with Culex abundance and WNV infection. The mean age of the horses in the study was 13.8 years (range 4–25 years). The 2004 seroconversion peak occurred after an increase in Cx. tarsalis abundance (Figure 1). In 2005 the seroconversion peak occurred after an increase in both Cx. tarsalis and Cx. pipiens populations (Figure 1).

An incidence density rate (IDR) of WNV infection (clinical and subclinical infections) was calculated for 2004 and 2005 to adjust for the open population and different horse-months at risk that each horse contributed to the study. For 2004, the WNV IDR was 0.0165 cases per horse-month (303 horse-months were contributed for 2004) or 0.198 cases per horse-year. For 2005, the WNV IDR was 0.075 cases per horse-month (240 horse-months were contributed by November 2005) or 0.9 cases per horse-year. Differences between the yearly seroprevalence rates were significant ($P < 0.005$), as determined by Poisson rates using the length of observation as the cumulative horse-months contributed for each year.

Median time to seroconversion, or the time at which half of the horses had seroconverted, was not determined for the 2004 data set because only 16% of the population seroconverted. For 2005, the median time for seroconversion was Month 8, or August (Table 1). The hazard ratio was 0.19; therefore, the estimated relative risk of WNV seroconversion in 2004 was 19% of the relative risk for WNV seroconversion in 2005 (95% CI 0.07, 0.42).

The risk of WNV infection for horses at the CEH was approximately 80% greater in 2005 than during 2004. The 2005 survival curve calculated using the results from the equine serology was in agreement with the estimated survival curve from a cross-sectional serologic study with the median time to seroconversion of 8 months.

Mosquito sampling. A total of 24,726 mosquitoes were collected (Table 2) during 1,256 CO$_2$ trap nights and 1,299 gravid trap nights over 3 years. The cumulative sampling effort among the 3 years was 433 CO$_2$ trap nights and 450 gravid trap nights over 26 weeks in 2003; 443 CO$_2$ trap nights and 445
gravid trap nights over 27 weeks in 2004; and 380 CO₂ trap nights and 404 gravid trap nights over 24 weeks in 2005. Differences in trap nights were due to weather variations and trap failures. The species collected consisted predominantly of *Cx. tarsalis* and *Cx. pipiens*, purportedly the main vectors of WNV in the region. The total number of *Cx. tarsalis* collected with CO₂-baited and gravid traps remained consistent for all 3 years (Table 2). In contrast, the number of *Cx. pipiens* differed each year regardless of trap type (Table 2).

Tukey’s test for multiple comparisons was conducted on the 2003–2005 *Culex* collections to detect differences in mean abundance across years. Abundance of female *Cx. tarsalis* and *Cx. pipiens* collected with CO₂ traps were normally distributed. The Kolomogorov–Smirnov test to accept normality was confirmed for *Cx. pipiens* in 2003 (*P* = 0.06), 2004 (*P* = 0.18), and 2005 (*P* = 0.9) and for *Cx. tarsalis* in 2003 (*P* = 0.37), 2004 (*P* = 0.27), and 2005 (*P* = 0.62). The number of *Cx. tarsalis* caught did not vary among years (*P* = 0.537), but that of *Cx. pipiens* did differ significantly (*P* < 0.0001). The gravid trap collections were not normally distributed. A Kruskal–Wallis test comparing gravid trap collections did not detect differences for *Cx. tarsalis* among the 3 years (*P* = 0.06) but did detect significant differences for *Cx. pipiens* (*P* < 0.0001).

In 2003, 11,763 mosquitoes were collected, of which 11,336 were tested and all were negative for arboviruses (Table 2).

### Table 1
Survival Analysis of 2004 and 2005 WNV seroconversion in nonvaccinated horses at the Center for Equine Health in Davis, CA

<table>
<thead>
<tr>
<th>Year (sample size)</th>
<th>2004 (N = 32)</th>
<th>2005 (N = 29)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median survival (in months)</td>
<td>n/a</td>
<td>8</td>
</tr>
<tr>
<td>Survival time (in months)</td>
<td>Survival proportion</td>
<td>Standard error</td>
</tr>
<tr>
<td>3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5</td>
<td>0.968</td>
<td>0.032</td>
</tr>
<tr>
<td>6</td>
<td>0.871</td>
<td>0.06</td>
</tr>
<tr>
<td>7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Figure 1.** *Culex tarsalis* (A, C) and *Culex pipiens* (B, D) abundance in dry-ice–baited (CO₂) and gravid traps and equine WNV seroconversions at the Center for Equine Health in Davis, CA, 2004 and 2005, respectively. Arrow indicates the week that the positive mosquito pool was collected.
agreeing with mosquitoes tested from throughout California north of the Tehachapi Mountains.\textsuperscript{39} In 2004, a total of 6,474 mosquitoes were collected, of which 6,353 were tested (Table 2). One Cx. pipiens pool that was collected from a gravid trap on September 3 was positive for WNV (Figure 1). The 2004 WNV MIR for Cx. pipiens was 0.22 (95% C.I. 0.01, 1.07) per 1,000 among females collected in gravid traps. During the week when the mosquito infection was detected, the MIR was 18.36 (95% C.I. 1.14, 108.19) per 1,000.

In 2005, a total of 6,488 female mosquitoes were collected, of which 6,164 were tested (Table 2). Two pools of female Cx. tarsalis collected in CO\textsubscript{2} traps on July 14 and 19 and 2 pools of female Cx. pipiens collected from gravid traps on July 28 and September 7 tested positive for WNV (Figure 1). For host-seeking female Cx. tarsalis, the yearly MIR was 1.98 per 1,000 (95% CI 0.36, 6.54) and for gravid Cx. pipiens the yearly MIR was 0.96 per 1,000 (95% CI 0.17, 3.13). Values for Cx. pipiens were not significantly different from 2004. MIRs during the periods when infection was detected were 12.99 (95% CI 2.50, 45.86) for Cx. tarsalis in mid-July. MIRs for Cx. pipiens were 9.55 (95% CI 0.57, 48.27) in late July and 19.10 (95% CI 1.17, 95.54) in early September.

\textbf{Climate variation.} The daily minimum and maximum temperatures from 2004 and 2005 for Davis, California, are plotted with the minimum temperature threshold for viral replication at 14.3°C\textsuperscript{9} (Figure 2). Nightly temperatures during

\begin{table}
\centering
\caption{Mosquito species trapped at the Center for Equine Health, Davis, CA, 2003–2005}
\begin{tabular}{lcccccc}
\hline
\textbf{Genus} & \textbf{Species} & \textbf{2003} & \textbf{2004} & \textbf{2005} & \textbf{2003} & \textbf{2004} & \textbf{2005} \\
\hline
Anopheles & freeborni & 58 & 80 & & 15 & 7 & 33 & 15 \\
 & franciscanus & 1 & – & 13 & 3 & – & 1 & – \\
Aedes & melaninum & 42 & – & 2 & – & 5 & – & 3 \\
 & sierrensis & 22 & 8 & 22 & 1 & 128 & 3 & 1 \\
 & pipiens & 1,503 & 8,275 & 791 & 4,190 & 3,240 & 1,848 & 1,848 \\
 & stigmatosa & 11 & 89 & 17 & 187 & 3 & 2 & 3 \\
 & tarsalis & 1,379 & 98 & 1,126 & 42 & 1,028 & 48 & 48 \\
Culiseta & incidens & 21 & 136 & – & 53 & 11 & 100 & 100 \\
 & inornata & 31 & 8 & 5 & – & 16 & 6 & 6 \\
Total & & 3,069 & 8,694 & 1,991 & 4,483 & 4,464 & 2,024 & 2,024 \\
\hline
\end{tabular}
\end{table}

\textbf{FIGURE 2.} Daily minimum and maximum temperatures for Davis, CA, 2004–2005, with minimum temperature for WNV replication at 14.3°C.\textsuperscript{9}
2004 consistently remained below this threshold until July, when warm night temperatures were associated with WNV activity. WNV activity occurred earlier during the 2005 WNV season, which corresponded with warm nightly temperatures that remained above 14°C as early as May. The average maximum daily temperature from May to the end of September 2004 was 32°C (95% CI 31.36, 32.7), and the average minimum temperature was 12.9°C (95% CI 12.56, 13.33). In 2005, the average maximum daily temperature during the same time was 31.8°C (95% CI 31.0, 32.6) and the average minimum temperature was 13.0°C (95% CI 12.63, 13.43). Using t-tests, differences detected between the overall temperatures from May to September of 2004 and 2005 were not significant for maximum and minimum temperatures ($P = 0.71$ and $P = 0.76$, respectively). However, when the maximum and minimum temperatures were compared separately for each month, significant differences were detected. The minimum temperatures in May of 2005 were significantly warmer than in 2004. In contrast, the maximum temperatures in June were significantly warmer in 2004 than 2005. The maximum and minimum temperatures were both warmer in July 2005 than in July of 2004.

**Blood-meal identification.** The total blood-engorged female mosquitoes tested were the result of 80 collections over 2 years of red box sampling (Table 3). Blood meals were identified from 8 mosquito species. Although the combined 2-year sample sizes for *Cx. tarsalis* and *Cx. pipiens* were approximately the same, 93 and 86, respectively, a significantly larger proportion ($P < 0.005$) of blood-meal sources from *Cx. tarsalis* (67% of the total tested) was identified by the ELISA compared with *Cx. pipiens* (19% of the total tested). Blood meals from 70 *Cx. pipiens* were identified, of which 79% fed on avian hosts and 21% fed on equines. Blood meals from 31 *Cx. tarsalis* were identified, of which 72% fed on equines, 25% on birds, and 3% on lagomorphs. Monthly avian-to-equine blood-meal ratios did not significantly differ for both *Cx. tarsalis* and *Cx. pipiens*. *Anopheles* spp. did not feed on birds but frequently fed on horses and lagomorphs. The only confirmed human blood meal was from an engorged *Aedes sierrensis*. Few humans worked after dark at the CEH, limiting human host availability for night-biting *Culex* species.

**DISCUSSION**

Prior to late June of 2004, WNV activity had not been detected in the Sacramento Valley. During the study period, high levels of WNV infection occurred in horses despite low numbers of mammalophagic mosquitoes and reputed bridge vectors such as *Aedes* spp. WNV infection was only detected in *Culex* spp. The low abundance and low WNV infection rates observed in mosquitoes in our study illustrated how, even though mosquito surveillance indicators were near or below the threshold of detection, the risk of transmission to horses remained high. Our data illustrate that WNV mosquito surveillance alone may underestimate the amount of circulating virus necessary for tangential transmission to horses, even with intensive sampling efforts. The equine infection rate (clinical and subclinical) was 62% in 2005 and higher than expected based on mosquito measures of abundance and infection. The apparent-to-inapparent ratio ranged from 1:4 in 2004 to 1:17 in 2005, and, although a large proportion (70–96%) of equines were thought to remain uninfected and susceptible after outbreaks of clinical equine illness, our results indicated that acquired herd immunity may have been underestimated and may have contributed to the subsidence of equine cases during the on-going epidemic.

During the emergence of WNV in the Sacramento Valley, infection rates in incidental hosts (both humans and horses) increased in a similar manner. Human case incidences in surrounding Solano and Yolo counties showed similar increases; it was 0.0 and 0.6 per 100,000 in 2004 and increased to 1.3 and 7.1 per 100,000 in 2005, respectively. Similarly, one clinical equine case, outside of our study, was reported from each county in 2004, but 16 were reported in Solano County and 14 in Yolo County in 2005. A human WNV outbreak occurred in both counties during 2006, and although no equine cases were reported, this may be reflective of an increase in equine vaccinations as well as naturally acquired immunity. Consequently, equine cases reported in subsequent years, especially in areas where WNV has been previously detected, may actually be indicative of high levels of WNV activity.

Higher incidence of WNV infection in the horses in 2005 was temporally associated with changes in temperature. Warm temperatures enable virus transmission by shortening the duration of the mosquito gonotrophic cycle, thereby increasing the vector–host contact rate and the duration of the extrinsic incubation period. Although the maximum daytime temperatures were significantly warmer for June in 2004, night temperatures for both 2004 and 2005 dropped below 14.3°C, the minimum threshold for viral replication in infected mosquitoes. This may have limited WNV transmission in June. WNV activity peaked in July of 2005, whereas the 2004 WNV activity did not peak until September, even though 1 horse seroconverted earlier in the summer. A possible explanation for this difference is that the May and July 2005 average minimum and maximum temperatures were significantly warmer than in 2004, perhaps enabling earlier virus amplification; however, further studies are needed to be more conclusive.

Results from the blood-meal analyses implicated both *Cx.*

<table>
<thead>
<tr>
<th>Blood-meal type</th>
<th>No. identified</th>
<th><em>Ae. melanimon</em></th>
<th><em>Ae. sierrensis</em></th>
<th><em>An. freeborni</em></th>
<th><em>An. franciscanus</em></th>
<th><em>Cx. pipiens</em></th>
<th><em>Cx. tarsalis</em></th>
<th><em>Cx. incidens</em></th>
<th><em>Cs. inornata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Avian</td>
<td>63 (24%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>55 (64%)</td>
<td>7 (7.5%)</td>
<td>–</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Horse</td>
<td>73 (27%)</td>
<td>–</td>
<td>1 (17%)</td>
<td>23 (68%)</td>
<td>9 (33%)</td>
<td>15 (17%)</td>
<td>23 (25%)</td>
<td>2 (11%)</td>
<td>–</td>
</tr>
<tr>
<td>Lagomorph</td>
<td>20 (7.5%)</td>
<td>1 (100%)</td>
<td>–</td>
<td>4 (12%)</td>
<td>14 (52%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Human</td>
<td>1 (0.5%)</td>
<td>–</td>
<td>1 (17%)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Unidentified</td>
<td>109 (41%)</td>
<td>–</td>
<td>4 (66%)</td>
<td>7 (20%)</td>
<td>4 (15%)</td>
<td>16 (19%)</td>
<td>62 (67%)</td>
<td>16 (89%)</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>266</td>
<td>1</td>
<td>6</td>
<td>34</td>
<td>27</td>
<td>86</td>
<td>93</td>
<td>18</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 3**

pipienti and Cx. tarsalis in the transmission of WNV to horses. While both species were abundant compared with other mosquito species at the study site and found naturally infected at a low rate, it was necessary to determine their blood-feeding patterns to indicate which species most likely transmitted WNV to horses. Although Cx. pipienti is considered to be mostly ornithophagic,\textsuperscript{45,46} a previous study that identified mammalian blood sources indicated Cx. pipienti tended to feed more on larger mammals than on small mammals.\textsuperscript{47} Our findings are important because we selectively collected blood-engorged female mosquitoes around an equine facility and identified Cx. pipienti feeding on horses, illustrating that, perhaps due to host availability, this species will bite and transmit WNV to horses.

In Colorado and California, human WNV cases tend to peak in July and August following the peak in the abundance of WNV-infected mosquitoes that occur in June and July. The number of cases may be amplified by the shift in Cx. tarsalis feeding from birds to mammals.\textsuperscript{48,49} The 2005 equine seroconversion rates in our study peaked in July. We were not able to detect seasonal shifts in feeding preferences from avian to equine hosts by Cx. pipienti or Cx. tarsalis, because our sample sizes were relatively small and possibly because collection of blood-engorged mosquitoes was restricted to June through October. However, the monthly ratios from June to October for Cx. pipienti feeding on avian hosts to equine hosts were approximately constant at 5:1. Blood-meal sources for a large proportion of Cx. tarsalis were not identified, perhaps reflecting opportunistic feeding by this species.\textsuperscript{23} A variety of potential mammalian hosts at the CEH study area, such as raccoons, skunks, squirrels, possums, coyote, etc., may have comprised the unidentified blood meals in Cx. tarsalis. However, most identified blood meals for Cx. tarsalis were from horses.

Genetic variation exists within Cx. pipienti populations within the Sacramento Valley and includes hybrids with Cx. quinquefasciatus,\textsuperscript{22} which may explain the higher proportion of Cx. pipienti feeding on equine hosts in this study than anticipated.\textsuperscript{50} Further genetic studies should be performed on Cx. pipienti from the Central Valley of California to determine if the hybrid zone between Cx. pipienti and Cx. quinquefasciatus is continually changing as was previously determined\textsuperscript{22} and if this genetic variation contributes to the increased risk for equine and human infection. Our results indicate that Cx. pipienti and Cx. tarsalis may have both been important vectors of WNV to horses in the Sacramento Valley during the equine outbreaks that occurred in 2004 and 2005.

We conclude that immunologically naive horses are more sensitive for detection of WNV transmission in our study area than mosquito sampling alone. Local chicken flocks that were used for surveillance in the surrounding counties did not detect WNV transmission in 2004 until September, even though chickens are thought to provide a sensitive means to early detection of arbovirus activity in California\textsuperscript{40,57}; this further illustrates how equine infections in Northern California were sensitive indicators of early-season transmission when WNV was first emerging in the area. Serologic surveillance is dependent on serial serum collections from horses with known infection history, can be expensive, and may be delayed until there is a diagnostic rise in antibody, thus impeding immediate decisions for public-health control measures. Trends in the epidemiologic curves for the 1999 and 2000 WNV outbreaks in the Northeast show equine cases occurring after human and wild bird cases.\textsuperscript{42} Previous viral activity in wild birds and mosquitoes had been identified in areas prior to equine outbreaks.\textsuperscript{43} Since initiation of the successful equine vaccination program, the number of susceptible horses and the corresponding decrease in the number of clinical equine cases from WNV infection has reduced the utility of using horses as sentinels. Moreover, there are ethical reasons for not using unprotected equines as sentinel animals to detect WNV transmission. Based on our results, however, when evidence of early-season equine infection is available, it should be considered a sensitive indicator of WNV activity and be used to accelerate vector control measures and veterinary vaccination programs to reduce the risk of WNV infection for horses and humans.

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