Phenotypic Variation among *Culex pipiens* Complex (Diptera: Culicidae) Populations from the Sacramento Valley, California: Horizontal and Vertical Transmission of West Nile Virus, Diapause Potential, Autogeny, and Host Selection

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Abstract. The vector competence and bionomics of *Culex pipiens* form *pipiens* L. and *Cx. pipiens* f. *molestus* Forskäl were evaluated for populations from the Sacramento Valley. Both f. *pipiens* and f. *molestus* females became infected, produced disseminated infections, and were able to transmit West Nile virus. Form *molestus* females also transmitted West Nile virus vertically to egg rafts and F₁ progeny, whereas f. *pipiens* females only transmitted to egg rafts. *Culex pipiens* complex from urban Sacramento blood-fed on seven different avian species and two mammalian species. Structure analysis of blood-fed mosquitoes identified K = 4 genetic clusters: f. *molestus*, f. *pipiens*, a group of genetically similar hybrids (Cluster X), and admixed individuals. When females were exposed as larvae to midwinter conditions in bioenvironmental chambers, 85% (N = 79) of aboveground *Cx. pipiens* complex females and 100% (N = 34) of underground f. *molestus* females did not enter reproductive diapause.

INTRODUCTION

Two important vectors of West Nile virus (WNV, *Flaviviridae: Flavivirus*) in California, *Culex pipiens* L. and *Culex quinquefasciatus* Say, are members of the *Cx. pipiens* complex that occur worldwide. Members of this complex exhibit little morphological variation, but have distinctive physiological and behavioral traits. Northern *Cx. pipiens* occupies aboveground habitats and enters a reproductive diapause,^{1,2} whereas southern *Cx. quinquefasciatus* occupies above- and belowground habitats,³ does not enter diapause, but rather may overwinter in a temperature-induced quiescence, with short periods of inactivity.^{4,5} Additionally, *Cx. pipiens* exists as two identical morphological forms, denoted *pipiens* and *molestus* Forskäl.⁶ *Culex pipiens* f. *molestus* inhabit underground environments, develop their first batch of eggs without a bloodmeal (autogeny), mate in confined spaces (stenogamous), and remain reproductively active (gonoactive) throughout the year.² In contrast, *Culex pipiens* f. *pipiens* develop in aboveground habitats, require a bloodmeal for egg development (anautozeny), mate in open areas (eurygamous), and undergo a reproductive diapause during winter. Genetic introgression between members of this complex in California, especially in the Central Valley, has made identification of populations using morphological and allozyme marker analysis^{7,8} unreliable.⁹

Culex pipiens and *Cx. quinquefasciatus* are competent vectors of WNV in California, although there is spatio-temporal variability.^{10–13} Both environmental and genetic factors may influence vector competence¹³ and impact the ability of populations to become infected and transmit virus.¹⁴ Although no study has evaluated f. *molestus* populations in California, European f. *molestus* are efficient vectors of WNV^{15,16}; however, autogenous populations rarely feed on blood until uniparous.^{1,17} Additionally, vertical transmission of WNV, the passage of virus from an infected parent to her offspring during egg deposition, has been shown in both field and experimental

populations of *Cx. pipiens* and *Cx. quinquefasciatus*.^{18–25} The ability of f. *molestus* populations to vertically transmit WNV has not been evaluated.

In North America, it is generally accepted that *Cx. pipiens* and *Cx. quinquefasciatus* females are primarily ornithophilic¹⁴; however, they also serve as important bridge vectors of WNV to humans.^{26–29} In contrast, f. *molestus* is considered to be mammalophilic, with humans frequently the primary host.⁶ It was hypothesized that hybridization between these forms may make *Cx. pipiens* a superior bridge vector of WNV to humans as mosquitoes would initially become infected by feeding on birds and then transmit the virus to humans.^{30–32} Recent studies found that *Cx. pipiens* females were more likely to feed on mammals if they had a higher genetic ancestry from f. *molestus*.^{33,34} However, three recent bloodmeal identification studies done in California showed that *Cx. pipiens* complex females fed infrequently on mammals, including humans, even in highly urbanized areas^{35–37}; however, these studies did not evaluate the genetic ancestry of the individual females tested.

Form *molestus* has been extensively studied in Europe, but relatively few populations have been found in the United States.^{32,33,38–42} Fonseca and others³⁰ found extensive hybridization (40%) between *pipiens* and *molestus* forms in the United States compared with European populations ($\leq 10\%$), and a more recent study⁴² found that autogenous populations in Santa Clara County, CA, were a genetic mix of f. *molestus* hybridized with *Cx. quinquefasciatus* and to a lesser extent f. *pipiens*. In contrast, populations from Chicago and New York had a low number of hybrids (8%) between forms.⁴³ Autogenous populations of *Cx. pipiens* recently were detected in catch basins and manholes in the Sacramento Valley of California,⁴⁴ Marin County,⁴⁵ and Santa Clara County,⁴² supporting the unpublished studies of Iltis (Iltis WG, unpublished dissertation) who reported widespread autogeny among *pipiens* populations throughout much of central California.

The role that f. *molestus* populations play in the overwintering of WNV is not well understood. In southern California, temperatures in underground storm drain systems remain relatively stable year round compared with aboveground temperatures, and these underground temperatures during winter may allow continued WNV transmission by

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Cx. quinquefasciatus.^{3,46} Additionally, a study conducted in northern California⁴² found that water temperatures in an underground location harboring *f. molestus* also were relatively constant during winter. These temperatures may allow WNV to replicate within infected females that remain gonotactically active during the unfavorable winter period, emerge during warm weather, and transmit virus.

Recently, the genetic structure of *Cx. pipiens* complex populations was evaluated in detail for our study sites in the Sacramento area of California.⁴⁷ Our current study compared the phenotypic traits of those populations including horizontal and vertical vector competence for WNV, diapause potential, autogeny status, and host selection for these autogenous (*f. molestus*) and anautogenous (*f. pipiens*) populations.

METHODS

Virus and mosquito strains. Mosquitoes were infected with a California strain of WNV isolated in 2004 from a Yellow-billed Magpie that died in Sacramento (CA04, GenBank accession no. DQ080059) and was passaged three times in Vero (African green monkey kidney) cells before experimentation. *Culex pipiens* complex mosquitoes used in the current research were from the populations characterized genetically by Kothera and others.⁴⁷ For experimental horizontal and vertical transmission studies, strains of *f. molestus* and *f. pipiens* were obtained from laboratory colonies established in 2010 by the Sacramento Yolo Mosquito and Vector Control District (SYMVCD) from specimens collected in Sacramento (38.576 N, -121.490 W) and Woodland (38.684 N, -121.780 W), CA, respectively. For experiments evaluating the genetic potential for diapause induction and autogeny, natural populations of *Cx. pipiens* complex mosquitoes from the Sacramento Valley were collected from two aboveground locations in Woodland and Davis [heronry] (38.603 N, -121.711 W and 38.673 N, -121.782 W) and one underground location in Old Sacramento (38.584 N, -121.504 W) that were described previously.⁴⁷ Additionally, *Cx. quinquefasciatus* and *Cx. pipiens* mosquitoes were obtained from Los Angeles (33.936 N, -118.065 W) and Shasta Counties (40.480 N, -122.347 W and 40.479 N, -122.326 W), respectively.⁴⁷ A colonized strain of *Cx. pipiens* from Oak Lawn, IL (41.719 N, -87.756 W) and field-collected *Culex stigmatosoma* Dyar and *Culex tarsalis* Coquillett from the Sacramento Valley (38.603 N, -121.711 W) were used as positive diapause controls.

Horizontal vector competence. Five- to 7-day-old *f. molestus* (uniparous females after initial autogenous oviposition) and *f. pipiens* females were starved (all sugar and water removed 24 hr before blood feeding), and then infected orally using a membrane feeder (Hemotek, *Discovery Workshops*, Accrington, Lancashire, U.K.). Stock virus with a titer of $10^{8.6}$ plaque-forming units (PFU) per mL was added to heparinized chicken blood in a 1:5 dilution. Mosquitoes were allowed to feed for up to 2 hr, anesthetized with CO₂, and engorged females transferred to 20.3 × 21 (H × Dia.) cm holding containers. To estimate mean bloodmeal titer, 10% of females that blood-fed were collected immediately after feeding and frozen at -80°C until later testing by plaque assay. Infected females were held at a photoperiod of 14:10 (L:D) hr, 26°C, and provided Craisins (Ocean Spray Cranberries Inc., Lakeville-Middleboro, MA) and distilled water for sustenance.

At 14 d post infection, mosquitoes were immobilized with triethylamine. Expectorate was collected by the capillary tube method⁴⁸ and transferred to cryovials containing 500 µL of mosquito diluent (Dulbecco's modified eagle medium, containing 5% penicillin, 0.4% amphotericin, and 20% fetal bovine serum); legs and bodies were stored separately in cryovials containing two 5 mm glass beads and 1 mL of mosquito diluent. Expectorate, legs, and bodies were frozen at -80°C until assayed for infectious virus by plaque assay.

Vertical transmission. Form *f. molestus* and *f. pipiens* females were infected and held as described previously. At 14 d post infection, mosquitoes were immobilized with CO₂ and transferred into individual 50-mL conical, screen-topped vials, after which 5–10 mL of distilled water was added for oviposition. Following oviposition, individual females were removed, anesthetized with triethylamine, and placed into cryovials for immediate testing for WN viral RNA. Only progeny from females testing positive for WN viral RNA were analyzed further. Within 24–48 hr after hatching, empty egg rafts from each female were placed into cryovials and frozen at -80°C for later testing. Larvae from WNV positive females were reared by family and fed a 1:1 by volume mixture of rabbit chow and fish food as needed until pupation. Emerging adults were held up to 5 d post emergence, separated into groups of 10 according to family, and frozen in cryovials at -80°C until later testing by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Bloodmeal identification. Blood-fed *Cx. pipiens* complex females were collected opportunistically resting around or in catch basins and a manhole in urban Sacramento using a hand-held aspirator (Ryobi 18 Volt One+ Hand Vac, One World Technologies, Inc., Anderson, SC, modified into a mosquito vacuum). A manhole in downtown Sacramento (38.585 N, -121.491 W), a catch basin ~1 km from the manhole (38.577 N, -121.496 W), and a catch basin by the Sacramento Zoo (38.540 N, -121.505 W), where autogenous females had been collected previously,⁴⁷ were sampled from June 2010 to April 2011. For each blooded female, the abdomen was separated from the head and thorax and the ovaries removed to determine parity. To prevent contamination, dissection tools were cleansed between specimens using DNA AWAY wipes (Molecular BioProducts, Inc., San Diego, CA). Abdomens were analyzed to determine the source of the bloodmeal using the methodology of Thiemann and others,⁴⁹ whereas head and thoraces were saved for microsatellite analysis (below). Briefly, DNA was extracted from each abdomen using the DNeasy 96 Blood & Tissue Kit (Qiagen, Valencia, CA), and the mitochondrial gene cytochrome *c* oxidase I (*COI*) was amplified from each sample by nested PCR. First, primers flanking *COI* were used to amplify the entire gene, and then the 658-bp “barcoding” region was amplified using vertebrate-specific primers.^{50,51} Host DNA was identified by sequencing the *COI* gene and using the “Identify Specimen” feature of the Barcode of Life Data Systems (BOLD; www.boldsystems.org).

Microsatellite analysis. Heads and thoraces of blood-fed *Cx. pipiens* complex specimens were sent to the Centers for Disease Control and Prevention (CDC) in Fort Collins, CO, for genetic analysis using microsatellite methodology previously described.⁴⁷ These specimens were compared with *Cx. pipiens* complex populations throughout California and a *Cx. pipiens* population from Grand Junction, CO.⁴⁷ Briefly, heads and thoraces of blood-fed *Cx. pipiens* complex females were

homogenized with BA-1 diluent and DNA was extracted from a portion of the sample and used in each subsequent PCR reaction. A panel of 17 microsatellite loci was used to generate a multilocus genotype for each individual. The panel consisted of two multiplexes of eight and nine markers each and the forward primer of each primer pair was fluorescently labeled for subsequent visualization on a Beckman-Coulter (Fullerton, CA) CEQ8000 sequencer. Cluster analysis was performed using the program Structure to determine the most likely number of clusters (K) using conditions described previously.⁴⁷ Individual assignments to each cluster (i.e., *q* values) were examined to determine which individuals and populations were assigned to each cluster; the number of clusters was determined by the previous Structure analysis. A value of *q* ≥ 0.80 was set as representative of a “pure” individual in a cluster, and individuals with *q* values < 0.80 were considered hybrids of one or more clusters.⁴⁷

Experimental studies. To evaluate the genetic potential for diapause induction, gravid and/or blood-fed *Cx. pipiens* complex females were collected from locations in the Sacramento Valley during the summer of 2012 (see above) and placed into cartons with water for oviposition. Form molestus females were collected as teneral adults by vacuum aspiration from a manhole and were allowed to develop their eggs autogenously. Egg rafts also were collected from *Cx. pipiens* from Shasta County, *Cx. quinquefasciatus* from Los Angeles County, *Cx. pipiens* from Illinois, and *Cx. stigmatosoma* and *Cx. tarsalis* from Yolo County. Egg rafts were hatched at room temperature *en masse* by location, and 1st instar larvae were divided randomly into two groups. Group one larvae were reared under midwinter conditions of 10:14 (L:D) hr and 16°C, whereas group two larvae were reared under mid-summer conditions of 14:10 (L:D) hr and 26°C. The immatures were reared in 21 × 12 cm (H × Dia.) mosquito breeders (BioQuip Products Co., Gardena, CA) and fed as described previously. Emerging adults were held on water moistened cotton balls and Craisins and up to 10 females from each location were removed at 7, 14, 21, and 28 d post emergence and frozen at -80°C until dissection.

Dissection protocol. Ovarian dissection procedures were modified from Giglioli⁵² as described previously⁴⁴; briefly, ovaries were excised at 40× under a dissecting microscope. One ovary was rinsed and dried on a template microscope slide and viewed under phase contrast at 100× to determine parity by examining coiling of the ovarian tracheoles⁵³; the second ovary was placed in a small drop of 1:1 of Gentian Violet and physiological saline (0.9%) and the ovarioles disrupted using minute pins. Individual follicles were examined at 200× and 400× and the primary follicles classified morphologically by size and the degree of vitellogenesis in the most mature follicles^{54,55}; the lengths of five representative primary and secondary follicles were measured at 200×. The length of the primary follicle was measured from the base of the ovariole to the joining of the secondary follicle. The length of the secondary follicle was then measured to the distal tip of the germarium. The primary follicle length and the ratio of the primary to secondary follicle length were used to determine ovarian status^{56,57}; fully degenerated or autogenously developed follicles (> stage II) were not measured. For blood-fed, field-collected parous females, the number of dilatations was determined using the methods of Polovodova.⁵⁸

Follicles in diapausing mosquitoes were at stage I (stages N-Ib of Kawai),⁵⁵ whereas anautogenous females in host-seeking arrest were at stage II (stage I-II, IIa,b). Females with follicles at stages III-V and therefore developed past the resting stage (stage II) were considered autogenous regardless of insemination status. To determine if mating had occurred, spermathecae were removed, crushed using forceps in a drop of distilled water, and spermatozoa visualized under a compound microscope at 400× to determine insemination.

Diagnostics. All viral assays were done under biosafety level-3 conditions at the Center for Vectorborne Diseases (CVEC) laboratory in accordance with Biological Use Authorization protocol 0873 approved by Environmental Health and Safety of the University of California Davis. Samples were homogenized by mixer mill (MM300, Retsch, Haan, Germany) and total RNA extracted using a MagMAX Express-96 system following manufacturer protocols (Life Technologies: Applied Biosystems [ABI]; Carlsbad, CA). Each extraction plate contained a positive control generated from cultured virus of known titer in PFU/mL and at least two negative controls (mosquito diluent). Extracted RNA was analyzed for the presence and quantity of WN viral RNA by TaqMan One-Step (ABI) real-time qRT-PCR using an ABI7900 platform (Life Technologies: Applied Biosystems [ABI]). Samples were tested in singleplex (one primer/probe set) using the TaqMan assay per manufacturer protocols. The singleplex reaction was specific for the envelope region of the viral genome (WN1)⁵⁹: (forward) 5'- TCA GCG ATC TCT CCA CCA AAG -3', (reverse) 5'- GGG TCA GCA CGT TTG TCA TTG -3', and (probe) 6FAM-TGC CCG ACC ATG GGA GAA GCT -BHQ-1. All qRT-PCR plates contained a standard curve generated from cultured virus of known titer (PFU/mL) and negative water controls. Samples with a cycle threshold (Ct) score < 40 were considered positive. Samples that were positive for WN viral RNA using WN1 were confirmed by a second qRT-PCR using probes/primers from the NS1 region of the viral genome (WN2)⁶⁰: (forward) 5'-GGC AGT TCT GGG TGA AGT CAA -3', (reverse) 5'-CTC CGA TTG TGA TTG CTT CGT -3', and (probe) Quasar 670-TGT ACG TGG CCT GAG ACG CAT ACC TTG T-BHQ-2.

Bloodmeal and mosquito body, leg, and expectorant titers were calculated by plaque assay⁶¹ on Vero cell monolayers in six-well tissue culture plates. Samples were serially diluted 10-fold and 200 µL of each dilution was added to confluent cell monolayers. Cells were incubated at 37°C and 5% CO₂ for 1 hr to allow virus attachment and entry. After incubation, cells were covered with a primary overlay (nutrient media, 1% agarose, and 3% sodium bicarbonate) and 48 hr after absorption a second overlay (nutrient media, 1% agarose, 3% sodium bicarbonate, and 3% neutral red) was applied. After 72 hr plaques were counted and virus concentrations calculated as PFUs/1.0 mL.

Statistical analysis. The vertical transmission rate (VTR) was defined as the percentage of infected females that transmitted WNV to their egg rafts or progeny, regardless of the proportion of progeny infected.⁶² This percentage was calculated by dividing the total number of infected females transmitting virus to their egg rafts or progeny by the total number of infected females that laid egg rafts that produced progeny. The minimum filial infection rate (MFIR) is the minimum number of mosquitoes infected with WNV/1,000 offspring and was calculated using the bias-corrected maximum likelihood

TABLE 1

Infection, dissemination, and transmission rates, with average titers for colonized strains of *Culex pipiens* f. molestus and *Cx. pipiens* f. pipiens females

Species	f. molestus	f. pipiens
No. tested	38	40
Infection rate* (%)	22/38 (57.9)	22/40 (55.0)
Titer†	10 ^{5.8±1.16}	10 ^{5.4±0.85}
Dissemination rate‡ (%)	15/22 (68.2)	7/22 (31.8)
Titer†	10 ^{3.1±1.40}	10 ^{4.2±0.94}
Transmission rate§ (%)	5/22 (22.7)	4/22 (18.2)
Titer†	10 ^{1.9±0.80}	10 ^{3.1±1.02}

*Number of females with bodies positive for WNV/total surviving.

†Average titer ± SD of positive samples, expressed in plaque forming units (PFU) per milliliter.

‡Number of mosquito legs positive for WNV divided by the total number infected.

§Number of mosquito expectorants positive for WNV divided by the total number infected.

estimate⁶³ with 95% confidence intervals (CIs). Mosquitoes of each group were tested in pools of ≤ 10 adults. Pearson χ^2 tests (Minitab 15, Minitab Statistical Software, Inc., State College, PA) were used to determine if phenotypic differences between f. molestus and f. pipiens were significant. Body, leg, and expectorant titers were compared between forms by using a 2-sample *t* test.

RESULTS

Horizontal vector competence. Plaque assays conducted on the infectious viral bloodmeal fed to colonized strains of f. molestus and f. pipiens females estimated the titer to be 10^{8.4±0.12} PFU/mL. In agreement, the mean titer of the undigested bloodmeal within five engorged f. molestus and f. pipiens females was 10^{5.4±0.22} and 10^{5.6±0.12} PFU/mL, respectively. A total of 39 f. molestus and 42 f. pipiens females fed on infectious blood, and 38 and 40 survived 14 d post infection, respectively. Infection, dissemination, and transmission rates with average body, leg, and expectorant titers for f. molestus and f. pipiens are shown in Table 1. Virus was only present in saliva of mosquitoes that had disseminated infections to the legs (and positive bodies). Overall, there were no significant differences between forms in the infection rate ($\chi^2 = 0.066$, degrees of freedom [df] = 1, *P* = 0.797) or transmission rate ($\chi^2 = 0.140$, df = 1, *P* = 0.709), however, the dissemination rate was significantly higher in f. molestus ($\chi^2 = 5.82$, df = 1, *P* = 0.016) compared with f. pipiens females. Similarly, there

were no significant differences between forms in average body (*T* = 1.24, df = 38, *P* = 0.221), leg (*T* = -2.03, df = 17, *P* = 0.058), or expectorant (*T* = -1.98, df = 5, *P* = 0.105) titers for infected females.

Vertical transmission. Plaque assays conducted on the infectious viral bloodmeal fed to colonized strains of f. molestus and f. pipiens females estimated the titer to be 10^{8.4±0.01} PFU/mL. Similarly, the mean titer of the undigested bloodmeal within 8 engorged f. molestus and 16 engorged f. pipiens females was 10^{5.7±0.11} and 10^{5.8±0.18} PFU/mL, respectively. Eighty f. molestus and 160 f. pipiens females fed on infectious blood, but only 22 females from each group survived 14 d post infection, tested positive for WN viral RNA by qRT-PCR, and laid viable egg rafts. Of the 22 positive f. molestus and f. pipiens females in each group, 77% (17 of 22) and 86% (19 of 22) had Ct scores < 20, respectively, indicating a high virus body titer (> 10⁵ PFU/mL) based on our standard curves Table 2.

The percentage of f. molestus and f. pipiens females passing virus vertically to their egg rafts was 32% (7 of 22) and 41% (9 of 22), respectively, and statistically similar ($\chi^2 = 0.393$, df = 1, *P* = 0.531) (Table 2). All egg rafts from infected females with Ct scores > 20 were negative. Only one of the 22 infected f. molestus females transmitted WNV vertically to her egg rafts and transstadially to her adult progeny; this female also had a high virus body titer (Ct score of 14.2, > 10⁷ PFU/mL). Virus was detected in one pool of 8 females and 2 males, with a Ct score of 18.5 (> 10⁶ PFU/mL), giving an overall vertical transmission rate of 4.5% (1 of 22). The overall MFIR was 1.2 (95% CL, 0.07–5.61) infected progeny/1,000 progeny tested (*N* = 863) from infected f. molestus females (Table 2). None of the 22 infected f. pipiens females' transstadially transmitted virus to their adult progeny.

Host selection and genetic ancestry. A total of 58 blood-fed *Cx. pipiens* complex females were collected resting in and around a manhole and two catch basins in urban Sacramento. Forty of these were collected from the manhole, 11 were collected from the catch basin in downtown Sacramento, and 7 were collected from the catch basin by the Sacramento Zoo. A total of 40 bloodmeal hosts were identified (Figure 1): 28 from the manhole, 7 from the downtown basin, and 5 from the Zoo. Of these, 16 females were uniparous and 3 females were multiparous each having two dilatations. During June to September 2010, House Finch (35% [6 of 17]), Mourning Dove, and American Robin (18% each [3 of 17]) comprised

TABLE 2

Experimental vertical transmission of West Nile viral RNA to eggs and adult progeny of infected *Culex pipiens* f. molestus and *Cx. pipiens* f. pipiens females

Ct score of infected females (viral titer)*	f. molestus				
	No. families tested	No. + egg rafts/ no. tested (%)	No. + families/ No. tested (VTR†)	Total positive pools/total tested (total no. of mosquitoes)	MFIR‡
< 20 (> 10 ⁵)	17	7/17 (41)	1/17 (5.9)	1/91 (700)	1.4/1000
> 20 (< 10 ⁵)	4	0/4 (0)	0/4 (0)	0/18 (134)	< 7.5/1000
Total	22	7/22 (32)	1/22 (4.5)	1/109 (863)	1.2/1000
Ct score of infected females (viral titer)*	f. pipiens				
	No. families tested	No. + egg rafts/ No. tested (%)	No. + families/ No. tested (VTR†)	Total positive pools/total tested (total no. of mosquitoes)	MFIR‡
< 20 (> 10 ⁵)	19	9/19 (47)	0/22 (0)	0/161 (1363)	< 0.7/1000
> 20 (< 10 ⁵)	3	0/3 (0)	0/3 (0)	0/22 (199)	< 5.0/1000
Total	22	9/22 (41)	0/22 (0)	0/183 (1562)	< 0.6/1000

*The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) Ct (cycle threshold) of infected females. West Nile viral titer expressed as plaque-forming units (PFU) per mL.

†Vertical transmission rate (VTR): the percentage of females transmitting virus to their progeny, regardless of the proportion of progeny infected.

‡Minimum filial infection rate (MFIR): minimum number of mosquitoes infected with WNV/1,000 offspring calculated using the bias-corrected maximum likelihood estimator (MLE).

FIGURE 1. Percentage of total identified bloodmeals from *Culex pipiens* complex females collected resting in and around a manhole and catch basins in Sacramento, CA, from June 2010 to April 2011. No blood-fed females were collected during Jan–Feb 2011.

the majority of *Cx. pipiens* complex feeds, whereas American Crow and Cedar Waxwing (43% each [3 of 7]) were most frequently fed-upon from October–December. No blood-fed *Cx. pipiens* complex females could be collected during January–February. A renewal in blood-feeding activity occurred in the spring (April–March) when 16 blood-fed females were collected and of these, 69% ($N = 11$) fed upon Cedar Waxwings. Overall, the most frequently fed-upon host during the sampling period was Cedar Waxwing, which comprised 40% (16 of 40) of the total bloodmeals. Only two mammal feeds were identified, one domestic cat during October–December and one dog during March–April.

Genetic ancestry could only be determined for 22 of the 40 blood-fed mosquitoes Table 3, most likely caused by DNA degradation. Structure analysis conducted on *Cx. pipiens* complex populations from California,⁴⁷ including the 22 blood-fed mosquitoes in this study, indicated four genetically distinct ancestry clusters ($K = 4$) using a cluster membership cutoff of 80% ($q \geq 0.80$): *Cx. quinquefasciatus*, *f. molestus*, a group of genetically similar individuals with genetic ancestry from *Cx. quinquefasciatus*, *f. pipiens*, and *f. molestus* (Cluster X), and admixed individuals. No *Cx. quinquefasciatus* were identified

from sites in this study. Overall, 50% (11 of 22) of the bloodmeals were from *f. molestus* females, 41% (9 of 22) from admixed females (those with a q value < 0.80 in any one cluster), and 5% (1 of 22) each from an *f. pipiens* and a Cluster X female. Of the two mammal feeds, one was identified from *f. molestus*; however, the majority of *f. molestus* feeds were from Cedar Waxwing (55% [6 of 11]). The three American Crow feeds were identified from females of admixed ancestry.

Diapause potential and autogeny. At 28 d post emergence, 90% (9 of 10) of *Cx. pipiens* complex progeny from Woodland and 100% ($N = 10$) of progeny from Davis had follicles $\geq$ stage I–II or degenerated (Figure 2) under experimental mid-winter conditions in a bioenvironmental chamber (Figure 3). Under summer conditions, 19% (15 of 80) of *Cx. pipiens* complex progeny from Woodland and Davis had degenerated follicles, significantly greater than 6% (5 of 79) under midwinter conditions ($\chi^2 = 5.58$, $df = 1$, $P = 0.018$). From 0% to 44% of anautogenous females from the Sacramento Valley appeared to arrest follicular development at stage I when dissected weekly. Additionally, 100% ($N = 34$) of F_1 progeny reared from *f. molestus* females collected from a manhole in Old Sacramento and reared under diapause conditions had ovarian follicles progress autogenously to stages III–V. Autogeny also was observed infrequently among the progeny of females collected from the aboveground location in Davis, with one female each dissected after 7 (midwinter conditions) and 14 (summer conditions) d post emergence developing eggs to stages III–V. Diapause also was not induced uniformly in *Cx. pipiens* females from Shasta County, because 20–70% of dissected females had follicles at the host-seeking arrest stage (stage II) or degenerated at each sampling period. Similar to females from the Sacramento Valley, 50% (20 of 40) of *Cx. pipiens* from Shasta County had degenerated follicles under summer conditions, significantly greater than 5% (2 of 40) under midwinter conditions ($\chi^2 = 20.31$, $df = 1$, $P < 0.0001$). *Culex quinquefasciatus* females from Greater Los Angeles County held under experimental summer and midwinter conditions showed essentially the same response, with 100% of females from each group with follicles developed to stage II or degenerated. Under summer conditions, a significantly greater proportion (40% [16 of 40]) of *Cx. quinquefasciatus* females had degenerated follicles than those under midwinter conditions (8% [3 of 40]; $\chi^2 = 11.67$, $df = 1$, $P = 0.001$). *Culex pipiens* progeny from a colony established from Oak Lawn, IL, and *Cx. tarsalis* and *Cx. stigmatosoma* progeny from Davis were reared concurrently in each bioenvironmental chamber.

TABLE 3

Number and percentage of bloodmeals by genetic ancestry of *Culex pipiens* complex females collected from urban Sacramento, CA, June 2010 to April 2011

Host	Admixed* (%)	<i>f. pipiens</i> † (%)	<i>f. molestus</i> ‡ (%)	Cluster X§ (%)
House Finch, <i>Carpodacus mexicanus</i>	2 (22.2)		2 (18.2)	
Mourning Dove, <i>Zenaidura macroura</i>	2 (22.2)		1 (9.1)	
Cooper's Hawk, <i>Accipiter cooperii</i>			1 (9.1)	
American Robin, <i>Turdus migratorius</i>				1 (100)
Cedar Waxwing, <i>Bombycilla cedrorum</i>	2 (22.2)	1 (100)	6 (54.5)	
American Crow, <i>Corvus brachyrhynchos</i>	3 (33.3)			
Domestic Cat, <i>Felis catus</i>			1 (9.1)	
Total Bloodmeals (%)	9 (40)	1 (5)	11 (50)	1 (5)

* Admixed specimens are those with $< 80\%$ ($q < 0.80$) membership in any one cluster by microsatellite analysis.

† Individuals with $\geq 80\%$ membership ($q \geq 0.80$) to *Cx. pipiens f. pipiens* by microsatellite analysis.

‡ Individuals with $\geq 80\%$ membership to *Cx. pipiens f. molestus* by microsatellite analysis.

§ Group of genetically similar ($q \geq 0.80$) individuals of hybrid origin (genetic ancestry from *f. pipiens*, *f. molestus*, and *Cx. quinquefasciatus*) by microsatellite analysis (Kothera and others⁴⁷).

FIGURE 2. Ovarian follicles from *Culex stigmatosoma* from Davis, CA (A, F), *Culex pipiens* from Oak Lawn, IL (B, G), *Cx. pipiens* from Shasta County, CA (C, H), *Cx. pipiens* from Davis and Woodland, CA (D, I), and *Culex quinquefasciatus* from Los Angeles, CA (E, J) held concurrently under midwinter (10:14 [L:D] hr, 16°C) and summer (14:10 [L:D] hr, 26°C) conditions in bioenvironmental chambers. Follicles A and B are diapausing at stage I, whereas follicles C–J are arrested at stage II or in various stages of degeneration.

Through 28 d post emergence under midwinter conditions, 100% of dissected females from each group had follicles arrested at development at stage I, indicating diapause (Figure 2). Conversely, under summer conditions, degenerated follicles were observed in 35% (14 of 40) of *Cx. pipiens* females from Oak Lawn. Although no *Cx. tarsalis* or *Cx. stigmatosoma* females survived past 21 d post emergence under experimental summer conditions, degeneration in the majority of follicles was only observed in one *Cx. stigmatosoma* female (3% [1 of 30]).

Insemination in small 21 × 12 cm cages under both midwinter and summer conditions occurred in 85% (60 of 71) of *f. molestus* females from Old Sacramento, reflecting their stenogamous mating behavior. Conversely, insemination rates were significantly less in 16% (13 of 80; $\chi^2 = 70.18$, df = 1, $P < 0.001$) and 24% (19 of 79; $\chi^2 = 54.83$, df = 1, $P < 0.001$) of aboveground *Cx. pipiens* complex females from Davis and Woodland, respectively. Similarly, insemination occurred in 19% (15 of 80) of *Cx. quinquefasciatus* females from Greater Los Angeles County. *Culex pipiens* from Shasta County and Oak Lawn had the lowest insemination rates, with only 1% (1 of 80) and 5% (4 of 80) of females containing sperm in their spermathecae, respectively. None of the *Cx. tarsalis* and *Cx. stigmatosoma* females was inseminated.

DISCUSSION

Vector competence. Colonized strains of *f. molestus* and *f. pipiens* from California were susceptible to infection with WNV, developed disseminated infections, and were able to transmit virus. Comparisons between forms showed greater

dissemination rates in *f. molestus* than *f. pipiens*, but there were no significant differences in infection rates, transmission rates, or titers. Our results are similar to a previous study evaluating *Cx. pipiens* from the Sacramento Valley that found 57% infected and 29% transmitting 14 d after feeding on $> 10^5$ PFU/mL of WNV.¹¹ Additional studies evaluating the vector competency of *Cx. quinquefasciatus* females in southern California reported infection rates between 28% and 66%,¹⁰ 44% and 76%,⁶⁴ 68%,¹¹ and 0% and 43%¹² when fed a high dose ($> 10^6$ PFU/mL) of WNV. Goddard and others¹⁰ found that 100% of *Cx. pipiens* females from Shasta County, CA, that fed on $\sim 10^7$ PFU/mL were infected after 14 d, whereas no females were infected 14 d after feeding on an equivalent infective dose in a similar study.¹² These studies also reported varying transmission rates among *Cx. pipiens* complex populations: 11–52% in southern California, 22–57% in the Central Valley, and 0–71% in northern California. In this study, dissemination rates were three and two times higher than transmission rates in *f. molestus* and *f. pipiens* females, respectively. This may be attributed to intrinsic factors such as salivary gland infection and escape barriers (i.e., legs were positive, but expectorant was negative).⁶⁵ Additionally, the amount of virus expectorated into capillary tubes by some females may have been less than the detectable limit by plaque assay,¹⁶ especially after our dilution into 500 μ L. Alternatively, these rates may have increased if the females were held for a longer time period. Overall, differences in the susceptibility of California *Cx. pipiens* complex mosquitoes to infection with WNV indicated that geographic, genetic and/or environmental factors, including possible co-infection with

FIGURE 3. Percentage of *Culex pipiens* complex mosquitoes from California and *Cx. pipiens* from Oak Lawn, IL, that were diapausing, in host-seeking arrest (quiescence), and autogenous after 7 (A), 14 (B), 21 (C), and 28 (D) days post emergence (dpe) in bioenvironmental chambers set to experimental midwinter (W = 10:14 [L:D] hr, 16°C) and summer (S = 14:10 [L:D] hr, 26°C) conditions. The control group combined both *Culex tarsalis* and *Culex stigmatosoma* females from Davis, CA, that produced identical results. The number of specimens dissected for each population and condition was 10, unless denoted otherwise in the figure.

Wolbachia,⁶⁶ may exist among California populations that affect their ability to become infected and transmit virus.

The first instance of vertical transmission of WNV by *f. molestus* was documented in this study, albeit at a low rate. Our vertical transmission rate of 4.5% is similar to a previous laboratory study using *Cx. pipiens* that reported a VTR of 5%⁶⁷ and our MFIR of 1.2 WNV infected progeny per 1,000 tested was similar to both field²³ and laboratory²² studies done with *Cx. pipiens* females from the eastern United States. Although WNV was not detected in adult progeny of colonized *f. pipiens* females, another colonized strain of *f. pipiens* from the Sacramento Valley, CA, successfully transmitted WNV to larval and adult progeny in a previous study.⁶⁸ In this study, *f. molestus* females were uniparous (after autogenous oviposition) before imbibing an infective bloodmeal, perhaps allowing increased dissemination of WNV to the reproductive tract, as vertical transmission rates have been shown to increase with the number of gonotrophic cycles completed by infected females.⁶⁷ The frequency of vertical transmission also may be influenced by rearing temperature and mosquito species and strains.^{67,69–71}

Host selection. Results from this study agreed with previous findings in the Sacramento Valley, documenting that *Cx. pipiens*

complex females feed primarily on birds and serve as WNV-amplifying, enzootic vectors.^{35,36,72} Montgomery and others³⁶ evaluated 23 non-rural (human habitation within 500 m) and 5 rural (no human inhabitants within 500 m) locations in the Sacramento Valley. One of his non-rural locations in downtown Sacramento was only 1 km and 0.5 km from the manhole and downtown catch basin sites used in this study, respectively. Montgomery and others³⁶ found that the highest percentages of bloodmeals in non-rural areas were from American Robins (22%, $N = 45$), followed by House Finches (17%, $N = 36$), and Mourning Doves (18%, $N = 37$). American Crows were also identified as hosts in that study, however comprised 1% ($N = 2$) of the total bloodmeals from non-rural sites. No mammal feeds were detected at non-rural locations and these only accounted for < 1% ($N = 3$) of bloodmeal hosts in rural locations. In this study, mammals accounted for 5% ($N = 2$) of bloodmeal hosts and no cases of human feeding were detected. We also identified House Finch, Mourning Dove, American Robin, and American Crow bloodmeals from engorged *Cx. pipiens* complex females. House Finches frequently were found seropositive for WNV and may be important maintenance hosts for the virus in urban areas of California.⁷³ Members of the family Corvidae, including

American Crow and Yellow-billed Magpie, are especially susceptible to WNV infection and develop extremely high viremias.^{74–77} Interestingly, Cedar Waxwings were the most frequently fed upon host, with the majority of bloodmeals detected during March–April. These birds are primarily winter residents in California,⁷⁸ but have been found infected with WNV.⁷⁴ Overall, 80% ($N = 32$) of total bloodmeals from *Cx. pipiens* complex females were from the avian order Passeriformes, perching birds that are important hosts in encephalitis virus transmission cycles.⁷⁷

Spielman¹ found that interbreeding between *pipiens* forms occurs primarily in the late summer to fall when anautogenous males outnumber autogenous males. He suggested that anautogenous populations amplify virus during spring and summer among susceptible avian hosts, with heterozygotes bridging virus to humans in the fall. Although studies have noted a significant association between *f. molestus* hybrid ancestry in *Cx. pipiens* individuals and a high proportion of mammalian³⁴ and human³³ blood feeding, no study has directly assessed blood feeding patterns from known *f. molestus* populations in the United States. Previous studies have noted frequent human biting by *f. molestus* females in underground railway tunnels in Europe,^{6,79} but did not directly evaluate host preferences of surface or subterranean populations. Additionally, two studies done in the Middle East found no differentiation in host preference between *f. molestus* and *f. pipiens* populations.^{80,81} Microsatellite analysis of blood-fed *Cx. pipiens* complex females collected from known autogenous populations in downtown Sacramento revealed four different genetic entities. The majority of bloodmeals were from *f. molestus* (50%), followed by those of admixed ancestry (41%). One bloodmeal each was from an *f. pipiens* female and a female that had hybrid genetic ancestry from *Cx. quinquefasciatus*, *f. pipiens*, and *f. molestus* (Cluster X).⁴⁷ Although one mammal feed was identified from an *f. molestus* female, the majority of bloodmeals were derived from avian hosts. Although *f. molestus* females and their hybrids fed on WNV-competent host species, autogenous forms usually do not become infected until their second gonotrophic cycle and this may diminish their importance as amplification vectors of WNV. Because *f. molestus* females rarely feed on blood until uniparous,^{1,17} collections of blood-fed individuals from known *f. molestus* populations were limited.

Systematics. Traditionally, *Cx. pipiens* complex mosquitoes found north of 39°N have been considered pure *Cx. pipiens* and those south of 36°N as pure *Cx. quinquefasciatus*, with hybrids identified between these two latitudes.^{7,82,83} Our recent study found extensive hybridization among *Cx. quinquefasciatus*, *f. pipiens*, *f. molestus*, and their hybrids throughout central California as far north as Shasta County (> 40°N).⁴⁷ Additionally, Strickman and Fonseca⁴² detected hybridization between *f. molestus* and *Cx. quinquefasciatus* and to a lesser extent *f. pipiens* in populations from Santa Clara County, CA. These genotypic findings agreed with phenotypic variation in diapause potential observed among *Cx. pipiens* complex populations from California. Quiescent, diapausing, and autogenous *Cx. pipiens* complex females from the northern Central Valley of California were able to persist during winter.⁴⁴ After 28 d post emergence in a bioenvironmental chamber under experimental midwinter conditions, the majority of anautogenous *Cx. pipiens* complex females were considered to be in quiescence. Autogenous

f. molestus females from underground locations in urban Sacramento developed mature eggs even under midwinter conditions, although *f. molestus* females were also detected in aboveground populations. As in previous studies,^{4,5} *Cx. quinquefasciatus* females did not undergo diapause, but entered a state of quiescence. Degenerated follicles were readily observed in the ovaries of nondiapausing members of the *Cx. pipiens* complex in California, a phenomenon observed previously in *Cx. pipiens*⁸⁴ and *Cx. quinquefasciatus*⁴ that were hormonally active and unable to imbibe a bloodmeal. Extensive genetic introgression coupled with mild temperatures in the northern Central Valley of California seem to permit variation in the overwintering strategies among *Cx. pipiens* complex populations, allowing a variety of overwintering phenotypes to persist during winter.⁴⁴

West Nile virus overwintering. Although *Cx. pipiens* complex populations in California appear to be competent vectors for WNV, the principal question is whether WNV can persist in these populations during winter. Both *f. molestus* and *f. pipiens*⁶⁸ from California are capable of vertically passing virus to their offspring during late summer. Because the majority of *Cx. pipiens* complex females in California overwinter in a state of quiescence or remain gonotrophic, WNV may persist in females that imbibed an infective bloodmeal during late summer/fall or in females infected vertically. Because reproductively active members of the *Cx. pipiens* complex were able to survive the winter period in the northern Central Valley,⁴⁴ infected females may also initiate transmission when temperatures are above the minimum threshold for virus replication. Nondiapausing *Cx. pipiens* females that were infected with WNV and held up to 42 d at 10°C were able to transmit after incubation at 26°C.⁸⁵ Our findings are in agreement with previous bloodmeal studies done in California, which suggest that *f. pipiens* and *f. molestus* mosquitoes in the Sacramento Valley of California frequently use competent passeriform hosts during spring. Because few blood-fed *f. molestus* females were collected in this study and these were not tested for WNV, future studies are necessary to evaluate their role in the maintenance and amplification of WNV in urban areas.

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REFERENCES

1. Spielman A, 2001. Structure and seasonality of nearctic *Culex pipiens* populations. *Ann N Y Acad Sci* 951: 220–234.
2. Vinogradova EB, 2000. *Culex pipiens pipiens* Mosquitoes: Taxonomy, Distribution, Ecology, Physiology, Genetics, Applied Importance and Control. Bulgaria: Pensoft, Sofia.
3. Su T, Webb JP, Meyer RP, Mulla MS, 2003. Spatial and temporal distribution of mosquitoes in underground storm drain systems in Orange County, California. *J Vector Ecol* 28: 79–89.
4. Reisen WK, Meyer RP, Milby MM, 1986. Overwintering studies on *Culex tarsalis* (Diptera: Culicidae) in Kern County, California: temporal changes in abundance and reproductive status with comparative observations on *C. quinquefasciatus* (Diptera: Culicidae). *Ann Entomol Soc Am* 79: 677–685.
5. Eldridge BF, 1968. The effect of temperature and photoperiod on blood-feeding and ovarian development in mosquitoes of the *Culex pipiens* complex. *Am J Trop Med Hyg* 17: 133–140.
6. Harbach RE, Harrison BA, Gad AM, 1984. *Culex* (*Culex*) *Molestus* Forskal (Diptera, Culicidae)—neotype designation, description, variation, and taxonomic Status. *Proc Entomol Soc Wash* 86: 521–542.
7. Tabachnick WJ, Powell JR, 1983. Genetic analysis of *Culex pipiens* populations in the central valley of California. *Ann Entomol Soc Am* 76: 715–720.
8. Urbanelli S, Silvestrini F, Reisen WK, DeVito E, Bullini L, 1997. Californian hybrid zone between *Culex pipiens pipiens* and *Cx. p. quinquefasciatus* revisited (Diptera: Culicidae). *J Med Entomol* 34: 116–127.
9. Cornel AJ, McAbee RD, Rasgon J, Stanich MA, Scott TW, Coetzee M, 2003. Differences in extent of genetic introgression between sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa. *J Med Entomol* 40: 36–51.
10. Goddard LB, Roth AE, Reisen WK, Scott TW, 2002. Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis* 8: 1385–1391.
11. Reisen WK, Barker CM, Fang Y, Martinez VM, 2008. Does variation in *Culex* (Diptera: Culicidae) vector competence enable outbreaks of West Nile virus in California? *J Med Entomol* 45: 1126–1138.
12. Vaidyanathan R, Scott TW, 2007. Geographic variation in vector competence for West Nile virus in the *Culex pipiens* (Diptera: Culicidae) complex in California. *Vector Borne Zoonotic Dis* 7: 193–198.
13. Kilpatrick AM, Fonseca DM, Ebel GD, Reddy MR, Kramer LD, 2010. Spatial and temporal variation in vector competence of *Culex pipiens* and *Cx. restuans* mosquitoes for West Nile virus. *Am J Trop Med Hyg* 83: 607–613.
14. Farajollahi A, Fonseca DM, Kramer LD, Marm Kilpatrick A, 2011. “Bird biting” mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. *Infect Genet Evol* 11: 1577–1585.
15. Tahori AS, Sterk VV, Goldblum N, 1955. Studies on the dynamics of experimental transmission of West Nile virus by *Culex molestus*. *Am J Trop Med Hyg* 4: 1015–1027.
16. Turell MJ, Mores CN, Dohm DJ, Komilov N, Paragas J, Lee JS, Shermuhemedova D, Endy TP, Kodirov A, Khodjaev S, 2006. Laboratory transmission of Japanese encephalitis and West Nile viruses by molestus form of *Culex pipiens* (Diptera: Culicidae) collected in Uzbekistan in 2004. *J Med Entomol* 43: 296–300.
17. Kassim NF, Webb CE, Russell RC, 2012. Is the expression of autogeny by *Culex molestus* Forskal (Diptera: Culicidae) influenced by larval nutrition or by adult mating, sugar feeding, or blood feeding? *J Vector Ecol* 37: 162–171.
18. Goddard LB, Roth AE, Reisen WK, Scott TW, 2003. Vertical transmission of West Nile virus by three California *Culex* (Diptera: Culicidae) species. *J Med Entomol* 40: 743–746.
19. Nasci RS, Savage HM, White DJ, Miller JR, Cropp BC, Godsey MS, Kerst AJ, Bennett P, Gottfried K, Lanciotti RS, 2001. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. *Emerg Infect Dis* 7: 742–744.
20. Reisen WK, Fang Y, Lothrop HD, Martinez VM, Wilson J, Oconnor P, Carney R, Cahoon-Young B, Shafii M, Brault AC, 2006. Overwintering of West Nile virus in Southern California. *J Med Entomol* 43: 344–355.
21. Anderson JF, Main AJ, 2006. Importance of vertical and horizontal transmission of West Nile virus by *Culex pipiens* in the Northeastern United States. *J Infect Dis* 194: 1577–1579.
22. Dohm DJ, Sardelis MR, Turell MJ, 2002. Experimental vertical transmission of West Nile virus by *Culex pipiens* (Diptera: Culicidae). *J Med Entomol* 39: 640–644.
23. Farajollahi A, Crans WJ, Bryant P, Wolf B, Burkhalter KL, Godsey MS, Aspen SE, Nasci RS, 2005. Detection of West Nile viral RNA from an overwintering pool of *Culex pipiens pipiens* (Diptera: Culicidae) in New Jersey, 2003. *J Med Entomol* 42: 490–494.
24. Bugbee LM, Forte LR, 2004. The discovery of West Nile virus in overwintering *Culex pipiens* (Diptera: Culicidae) mosquitoes in Lehigh County, Pennsylvania. *J Am Mosq Control Assoc* 20: 326–327.
25. McAbee RD, Green EN, Holeman J, Christiansen J, Frye N, Dealey K, Mulligan FS 3rd, Brault AC, Cornel AJ, 2008. Identification of *Culex pipiens* complex mosquitoes in a hybrid zone of West Nile virus transmission in Fresno County, California. *Am J Trop Med Hyg* 78: 303–310.
26. Kilpatrick AM, Kramer LD, Campbell SR, Alleyne EO, Dobson AP, Daszak P, 2005. West Nile virus risk assessment and the bridge vector paradigm. *Emerg Infect Dis* 11: 425–429.
27. Hamer GL, Kitron UD, Goldberg TL, Brawn JD, Loss SR, Ruiz MO, Hayes DB, Walker ED, 2009. Host selection by *Culex pipiens* mosquitoes and West Nile virus amplification. *Am J Trop Med Hyg* 80: 268–278.
28. Savage HM, Aggarwal D, Apperson CS, Katholi CR, Gordon E, Hassan HK, Anderson M, Charnetzky D, McMillen L, Unnasch EA, Unnasch TR, 2007. Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002–2003. *Vector Borne Zoonotic Dis* 7: 365–386.
29. Hamer GL, Kitron UD, Brawn JD, Loss SR, Ruiz MO, Goldberg TL, Walker ED, 2008. *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *J Med Entomol* 45: 125–128.
30. Fonseca DM, Keyghobadi N, Malcolm CA, Mehmet C, Schaffner F, Mogi M, Fleischer RC, Wilkerson RC, 2004. Emerging vectors in the *Culex pipiens* complex. *Science* 303: 1535–1538.
31. Savage HM, Ceianu C, Nicolescu G, Karabatsos N, Lanciotti R, Vladimirescu A, Laiv L, Ungureanu A, Romanca C, Tsai TF, 1999. Entomologic and avian investigations of an epidemic of West Nile fever in Romania in 1996, with serologic and molecular characterization of a virus isolate from mosquitoes. *Am J Trop Med Hyg* 61: 600–611.
32. Savage HM, Kothera L, 2012. The *Culex pipiens* complex in the Mississippi River Basin: identification, distribution, and bloodmeal hosts. *J Am Mosq Control Assoc* 28(4s): 93–99.
33. Kilpatrick AM, Kramer LD, Jones MJ, Marra PP, Daszak P, Fonseca DM, 2007. Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. *Am J Trop Med Hyg* 77: 667–671.
34. Huang S, Hamer GL, Molaei G, Walker ED, Goldberg TL, Kitron UD, Andreadis TG, 2009. Genetic variation associated with mammalian feeding in *Culex pipiens* from a West Nile virus epidemic region in Chicago, Illinois. *Vector Borne Zoonotic Dis* 9: 637–642.
35. Thiemann TC, Lemenager DA, Kluh S, Carroll BD, Lothrop HD, Reisen WK, 2012. Spatial variation in host feeding patterns of *Culex tarsalis* and the *Culex pipiens* complex (Diptera: Culicidae) in California. *J Med Entomol* 49: 903–916.
36. Montgomery MJ, Thiemann T, Macedo P, Brown DA, Scott TW, 2011. Blood-feeding patterns of the *Culex pipiens* complex in Sacramento and Yolo Counties, California. *J Med Entomol* 48: 398–404.
37. Molaei G, Cummings RF, Su T, Armstrong PM, Williams GA, Cheng M-L, Webb JP, Andreadis TG, 2010. Vector-host

- interactions governing epidemiology of West Nile virus in Southern California. *Am J Trop Med Hyg* 83: 1269–1282.
38. Huang SM, Molaei G, Andreadis TG, 2008. Genetic insights into the population structure of *Culex pipiens* (Diptera: Culicidae) in the northeastern United States by using microsatellite analysis. *Am J Trop Med Hyg* 79: 518–527.
 39. Kent RJ, Harrington LC, Norris DE, 2007. Genetic differences between *Culex pipiens* f. molestus and *Culex pipiens pipiens* (Diptera: Culicidae) in New York. *J Med Entomol* 44: 50–59.
 40. McAbee RD, Kang KD, Stanich MA, Christiansen JA, Wheelock CE, Inman AD, Hammock BD, Cornel AJ, 2004. Pyrethroid tolerance in *Culex pipiens pipiens* var molestus from Marin County, California. *Pest Manag Sci* 60: 359–368.
 41. Mutebi JP, Savage HM, 2009. Discovery of *Culex pipiens pipiens* form molestus in Chicago. *J Am Mosq Control Assoc* 25: 500–503.
 42. Strickman D, Fonseca DM, 2012. Autogeny in *Culex pipiens* complex mosquitoes from the San Francisco Bay Area. *Am J Trop Med Hyg* 87: 719–726.
 43. Kothera L, Godsey M, Mutebi J-P, Savage HM, 2010. A comparison of aboveground and belowground populations of *Culex pipiens* (Diptera: Culicidae) mosquitoes in Chicago, Illinois, and New York City, New York, using microsatellites. *J Med Entomol* 47: 805–813.
 44. Nelms BM, Macedo PA, Kothera L, Savage HM, Reisen WK, 2013. Overwintering biology of *Culex* mosquitoes (Diptera: Culicidae) in the Sacramento Valley of California. *J Med Entomol* 50: 773–790.
 45. Lee Y, Seifert SN, Nieman CC, McAbee RD, Goodell P, Fryxell RT, Lanzaro GC, Cornel AJ, 2012. High degree of single nucleotide polymorphisms in California *Culex pipiens* (Diptera: Culicidae) sensu lato. *J Med Entomol* 49: 299–306.
 46. Harbison JE, Metzger ME, Walton WE, Hu R, 2009. Evaluation of factors for rapid development of *Culex quinquefasciatus* in belowground stormwater treatment devices. *J Vector Ecol* 34: 182–190.
 47. Kothera L, Nelms BM, Reisen WK, Savage HM, 2013. Population genetic and admixture analyses of *Culex pipiens* complex (Diptera: Culicidae) populations in California, United States. *Am J Trop Med Hyg* 89: 1154–1167.
 48. Aitken T, 1977. An *in vitro* feeding technique for artificially demonstrating virus transmission by mosquitoes. *Mosq News* 37: 130–133.
 49. Thiemann TC, Brault AC, Ernest HB, Reisen WK, 2012. Development of a high-throughput microsphere-based molecular assay to identify 15 common bloodmeal hosts of *Culex* mosquitoes. *Mol Ecol Resources* 12: 238–246.
 50. Cooper J, Sykes G, King S, Cottrill K, Ivanova N, Hanner R, Ikononi P, 2007. Species identification in cell culture: a two-pronged molecular approach. *In vitro Cell Dev-An* 43: 344–351.
 51. Ivanova NV, Dewaard JR, Hebert PD, 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Mol Ecol Notes* 6: 998–1002.
 52. Giglioli M, 1963. Aids to ovarian dissection for age determination in mosquitoes. *Mosq News* 23: 156–159.
 53. Detinova TS, 1962. Age-grouping methods in Diptera of medical importance with special reference to some vectors of malaria. *Monogr Ser World Health Organ* 47: 13.
 54. Clements A, Boocock M, 1984. Ovarian development in mosquitoes: stages of growth and arrest, and follicular resorption. *Physiol Entomol* 9: 1–8.
 55. Kawai S, 1969. Studies on the follicular development and feeding activity of the females of *Culex tritaeniorhynchus* with special reference to those in autumn. *Trop Med* 11: 145–169.
 56. Reisen WK, Meyer RP, Milby MM, 1986. Overwintering studies on *Culex tarsalis* (Diptera, Culicidae) in Kern County, California—survival and the experimental induction and termination of diapause. *Ann Entomol Soc Am* 79: 664–673.
 57. Spielman A, Wong JA, 1973. Studies on autogeny in natural populations of *Culex pipiens*. III. Midsummer preparation for hibernation in anautogenous populations. *J Med Entomol* 10: 319–324.
 58. Polovodova V, 1949. The determination of the physiological age of female *Anopheles*, by the number of gonotrophic cycles completed. *Med Parazitol Parazitarn Bolezni* 18: 352–355.
 59. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT, 2000. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol* 38: 4066–4071.
 60. Shi PY, Kramer LD, 2003. Molecular detection of West Nile virus RNA. *Expert Rev Mol Diagn* 3: 357–366.
 61. Kramer LD, Wolfe TM, Green EN, Chiles RE, Fallah H, Fang Y, Reisen WK, 2002. Detection of encephalitis viruses in mosquitoes (Diptera: Culicidae) and avian tissues. *J Med Entomol* 39: 312–323.
 62. Turell MJ, 1988. Horizontal and vertical transmission of viruses by insect and tick vectors. Monath, TP, ed. *The Arboviruses: Epidemiology and Ecology*. Volume 1. Boca Raton, FL: CRC, 127–152.
 63. Biggerstaff BJ, 2003. *PooledInfRate: A Microsoft Excel Add-In to Compute Prevalence Estimates from Pooled Samples*. Fort Collins, CO: Centers for Disease Control and Prevention.
 64. Reisen WK, Fang Y, Martinez VM, 2005. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. *J Med Entomol* 42: 367–375.
 65. Hardy JL, Houk EJ, Kramer LD, Reeves WC, 1983. Intrinsic factors affecting vector competence of mosquitoes for arboviruses. *Annu Rev Entomol* 28: 229–262.
 66. Rasgon JL, Scott TW, 2003. *Wolbachia* and cytoplasmic incompatibility in the California *Culex pipiens* mosquito species complex: parameter estimates and infection dynamics in natural populations. *Genetics* 165: 2029–2038.
 67. Anderson JF, Main AJ, Delroux K, Fikrig E, 2008. Extrinsic incubation periods for horizontal and vertical transmission of West Nile virus by *Culex pipiens pipiens* (Diptera: Culicidae). *J Med Entomol* 45: 445–451.
 68. Nelms BM, Fechter-Leggett E, Carroll B, Macedo P, Klueh S, Reisen WK, 2013. Experimental and natural vertical transmission of West Nile virus by California *Culex* (Diptera: Culicidae) mosquitoes. *J Med Entomol* 50: 371–378.
 69. Anderson JF, Main AJ, Cheng G, Ferrandino FJ, Fikrig E, 2012. Horizontal and vertical transmission of West Nile virus genotype NY99 by *Culex salinarius* and genotypes NY99 and WN02 by *Culex tarsalis*. *Am J Trop Med Hyg* 86: 134–139.
 70. Hayes CG, Baker RH, Baqar S, Ahmed T, 1984. Genetic variation for West Nile virus susceptibility in *Culex tritaeniorhynchus*. *Am J Trop Med Hyg* 33: 715–724.
 71. Baqar S, Curtis GH, Murphy JR, Watts DM, 1993. Vertical transmission of West Nile virus by *Culex* and *Aedes* species mosquitoes. *Am J Trop Med Hyg* 48: 757–762.
 72. Thiemann TC, Reisen WK, 2012. Evaluating sampling method bias in *Culex tarsalis* and *Culex quinquefasciatus* (Diptera: Culicidae) bloodmeal identification studies. *J Med Entomol* 49: 143–149.
 73. Kwan JL, Klueh S, Madon MB, Reisen WK, 2010. West Nile virus emergence and persistence in Los Angeles, California, 2003–2008. *Am J Trop Med Hyg* 83: 400–412.
 74. Wheeler SS, Barker CM, Fang Y, Armijos MV, Carroll BD, Husted S, Johnson WO, Reisen WK, 2009. Differential impact of West Nile virus on California birds. *Condor* 111: 1–20.
 75. Reisen W, Brault AC, 2007. West Nile virus in North America: perspectives on epidemiology and intervention. *Pest Manag Sci* 63: 641–646.
 76. Brault AC, Langevin S, Bowen RA, Panella NA, Biggerstaff BJ, Miller BR, Komar N, 2004. Differential virulence of West Nile strains for American Crows. *Emerg Infect Dis* 10: 2161–2168.
 77. Komar N, Langevin S, Hinten S, Nemeth NM, Edwards E, Hettler D, Davis BS, Bowen RA, Bunning M, 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis* 9: 311–322.
 78. Witmer MC, Mountjoy DJ, Elliot L, 1997. Cedar waxwing (*Bombicilla cedrorum*). Poole A, ed. *The Birds of North America Online*. Ithaca, NY: Cornell Lab of Ornithology.
 79. Byrne K, Nichols RA, 1999. *Culex pipiens* in London underground tunnels: differentiation between surface and subterranean populations. *Heredity* 82: 7–15.

80. Nudelman S, Galun R, Kitron U, Spielman A, 1988. Physiological characteristics of *Culex pipiens* populations in the Middle East. *Med Vet Entomol* 2: 161–169.
81. Villani F, Urbanelli S, Gad A, Nudelman S, Bullini L, 1986. Electrophoretic variation of *Culex pipiens* from Egypt and Israel. *Biol J Linn Soc Lond* 29: 49–62.
82. Barr AR, 1967. Occurrence and distribution of the *Culex pipiens* complex. *Bull World Health Organ* 37: 293–296.
83. Iltis WG, 1966. *Biosystematics of the Culex pipiens Complex in Northern California*. Davis, CA: University of California-Davis.
84. Spielman A, Wong J, 1973. Environmental control of ovarian diapause in *Culex pipiens*. *Ann Entomol Soc Am* 66: 905–907.
85. Dohm DJ, Turell MJ, 2001. Effect of incubation at overwintering temperatures on the replication of West Nile virus in New York *Culex pipiens* (Diptera: Culicidae). *J Med Entomol* 38: 462–464.