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VECTOR/PATHOGEN/HOST INTERACTION, TRANSMISSION

Experimental and Natural Vertical Transmission of West Nile Virus by California *Culex* (Diptera: Culicidae) Mosquitoes

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ABSTRACT *Culex* (Diptera: Culicidae) mosquitoes, the primary summer vectors of West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV), also may serve as overwintering reservoir hosts. Detection of WN viral RNA from larvae hatched from eggs deposited by infected females during late summer and fall may provide evidence for the vertical passage of WNV to overwintering cohorts. To determine whether vertical transmission to the overwintering generation occurs in populations of *Culex* mosquitoes throughout California, larvae from naturally infected females were tested by family for WN viral RNA by real-time quantitative reverse transcription-polymerase chain reaction during August through October 2011. Viral RNA was detected in 34 of 934 *Culex tarsalis* Coquillett and *Cx. pipiens* complex females that laid viable egg rafts. From these egg rafts, first-instar larvae from nine families tested positive, yielding an overall field vertical transmission rate of 26% (n = 34). To determine whether the WNV may be lost transtadially during development to the adult stage, first-instar larvae and adult progeny from experimentally infected *Cx. pipiens* complex females were assessed for the presence and quantity of WN viral RNA. Most (≈75%) WNV infections were lost from positive families during larval development to the adult stage. In field and laboratory studies, only infected mothers with mean cycle threshold scores ≤20 vertically transmitted WNV to larval progeny, adult progeny, or both. In summary, vertical transmission of WNV was detected repeatedly in naturally infected *Culex* mosquitoes collected throughout California during late summer and fall, with females having high titered infections capable of passing WNV onto their progeny destined for overwintering.

KEY WORDS *Culex*, West Nile virus, overwintering, vertical transmission

West Nile virus (family *Flaviviridae,* genus *Flavivirus,* WNV) was first detected in the United States (New York) in 1999, and by 2003 it had spread from New York to California (Lanciotti et al. 1999, Reisen et al. 2004), where it is now endemic. WNV amplifies during the summer through horizontal transmission between ornithophagic *Culex* mosquitoes and passerine birds. Humans and horses are tangentially infected and serve as dead-end hosts because they do not produce sufficient viremias to infect mosquitoes (Weaver and Reisen 2010). *Culex tarsalis* Coquillett and members of the *Culex pipiens* complex are competent and efficient laboratory vectors of WNV (Goddard et al. 2002, Reisen et al. 2005) and are considered to be the principle rural and urban vectors, respectively, in California (Reisen et al. 2006b). In addition to their role as horizontal vectors, they also may serve as overwintering reservoir hosts. Vertical transmission (the passage of virus from an infected female to her progeny) by *Culex* mosquitoes may provide a mechanism for WNV persistence through the winter and subsequent horizontal transmission and amplification of virus in the spring (Reeves 1961, Rosen 1987, Reisen et al. 2006a, Reisen and Brault 2007).

The vertical passage of WNV from naturally infected *Culex* females to their progeny, which successfully overwintered and subsequently transmitted virus horizontally after diapause termination, has been demonstrated in the laboratory (Anderson and Main 2006). In addition, WNV has been isolated from overwintering *Culex* adults in the field (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005, Andreadis et al. 2010, Unlu et al. 2010). Because females destined for diapause purportedly do not blood feed (Mitchell and Briegel 1989), these findings suggest that vertical transmission of WNV must occur repeatedly in natural populations. However, WNV has never been recovered from diapausing adult females during the winter in California, although evidence of vertical transmission to *Culex quinquefasciatus* Say (Reisen et al. 2006a) and *Cx. tarsalis* (W.K.R., unpublished) adults reared from field-collected immatures has been found. At northern latitudes, female *Culex* are induced to enter reproductive diapause after larvae are ex-

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posed to shortening day lengths and cooling water temperatures (Eldridge 1987). Emerging females that are programmed for diapause do not take bloodmeals (Bellamy and Reeves 1963, Mitchell 1981), so the most likely means by which a diapausing, nulliparous female may become infected with WNV is through vertical transmission.

Previous winter isolates of WNV were recovered in locations where researchers collected large numbers of mosquitoes, such as in abandoned military bunkers (Nasci et al. 2001, Farajollahi et al. 2005). Difficulty in obtaining large sample sizes of overwintering *Culex* females from field collections may be one reason for the scant number of winter isolates of WNV from mosquitoes, especially in California. Furthermore, collection and rearing of sufficient numbers of immature mosquitoes during fall and winter have proven to be both a time- and space-limiting process. To circumvent these difficulties, we examined the ability of naturally infected *Culex pipiens* L., *Cx. quinquefasciatus,* and *Cx. tarsalis* females collected at foci of WNV activity to vertically transmit WNV to first-instar larvae. Testing female *Culex* mosquitoes and their progeny from a focus of virus activity maximized the probability of finding naturally infected females and therefore evidence of vertical transmission. Detection of West Nile (WN) viral RNA in larvae from infected females during late summer and fall may provide evidence of vertical passage of WNV into overwintering cohorts.

Although the exact mechanism of vertical transmission of flaviviruses is not well understood, it has been suggested to occur during oviposition (Rosen 1988). Testing older larvae and pupae for WNV has been done in previous vertical transmission investigations (Baqar et al. 1993, Turell et al. 2001); however, no study has directly compared vertical transmission rates of WNV between first-instar larvae and adult progeny. The current study compared vertical transmission rates between egg rafts, first-instar larvae, and adult progeny by using a colony of *Cx. pipiens* established from a highly endemic area of WNV activity in California.

The current research explores the notion that locations with active horizontal transmission during late summer and fall also should harbor winter populations of vertically infected mosquitoes and that these females terminate diapause and possibly transmit virus horizontally when temperatures warm to above the thermal minimum for WNV replication during the following winter and spring (≈14°C; Reisen et al. 2006b). In addition, Goddard et al. (2003) suggested that vertically infected mosquitoes may augment virus amplification during summer when horizontal transmission is at its peak. Identifying locations that harbor vertically transmitting mosquitoes and estimating the frequency of transmission in those locations may help local vector control agencies plan and implement more effective mosquito and WNV control strategies the following season.

Materials and Methods

Vertical Transmission. The frequency of vertical transmission to first-instar larvae was studied in natural populations and in the laboratory.

Field Investigations. Areas with recurrent, late season WNV transmission as detected by multiple positive pools of *Culex* mosquitoes were chosen during August–October 2011 in collaboration with the Greater Los Angeles County Vector Control District (VCD) in Los Angeles County (two locations): the Arbovirus Field Station in Kern County (five locations),and the Sacramento-Yolo Mosquito and Vector Control District (SYMVCD) in Sacramento County (four locations). Data from Sacramento County were summarized previously by Fechter-Leggett et al. (2012) but are included here for comparison. At each location, *Cx. pipiens* complex mosquitoes (*Cx. quinquefasciatus* from Kern and Los Angeles, *Cx. pipiens* L. mixed with form *molestus* Forskål, and *Cx. quinquefasciatus* from Sacramento; Kothera et al. 2012) were collected using gravid traps (Cummings 1992). Dry ice-baited suction traps (CO_2 traps) were used to collect host-seeking *Cx. tarsalis* mosquitoes in Kern County, and these mosquitoes were subsequently fed on young chickens (University of California [UC] Davis Institutional Animal Care and Use Committee protocol 15886). *Cx. pipiens* complex and *Cx. tarsalis* females were anesthetized with CO_2 and identified to species. Gravid *Cx. pipiens* complex females were immediately transferred individually into vials for oviposition, whereas *Cx. tarsalis* females were transferred into vials after feeding on young chickens. Water was then added to each vial for egg raft deposition.

Adult females were removed after oviposition, anesthetized with triethylamine (TEA), and then the bodies were stored in cyrovials at −80°C for later virus testing. Vials were monitored for the presence of first-instar larvae, and within 24 h after hatching, empty egg rafts and larvae kept separate by family were removed from oviposition vials. Larvae were strained using vacuum filtration to remove excess water and frozen at −80°C for later testing for WNV. Only egg rafts from SYMVCD were saved in cryovials at −80°C for later virus testing.

Laboratory Experiments. All laboratory investigations were done under BSL-3 conditions at the UC Davis Center for Vectorborne Diseases (CVEC) laboratory approved for use of WNV under Biological Use Authorization 0873 by the UC Davis Institutional Biosafety Committee. *Cx. pipiens* complex mosquitoes were obtained from an anautogenous colony established by the SYMVCD from specimens collected in Elk Grove, CA (38.426N, −121.383W), in 2010; this site was used in field studies during 2011. Mosquitoes were infected with a strain of WNV isolated in 2004 from a yellow-billed magpie in Sacramento (CA04, GenBank accession DQ080059) and passaged three times in Vero (African green monkey kidney) cells before experimentation. Three- to 5-d-old females were starved (all sugar and water removed 24 h before blood feeding) and then infected orally using a mem-

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brane feeder (Hemotek, Discovery Workshops, Accrington, Lancashire, United Kingdom). Stock virus with a titer of $10^{8.4}$ plaque-forming units (PFUs)/ml was added to heparinized chicken blood in a 1:5 dilution. Mosquitoes were allowed to feed for up to 2 h, anesthetized with CO_2, and then the engorged females were transferred to 20.3- by 21-cm (height by diameter) holding containers in groups of ≤100. To calculate 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 26°C, ≈50% RH, and a photoperiod of 14:10 (L:D) h and provided Craisins (Ocean Spray Cranberries Inc., Lakeville-Middleboro, MA) and distilled water for sustenance. After 15 d postinfection, mosquitoes were immobilized with CO_2 and transferred into individual 50-ml conical, screen-topped vials to which 5–10 ml of distilled water was added for oviposition.

After oviposition, individual females were removed, anesthetized with TEA, and placed into cryovials for immediate testing for WN viral RNA. Only progeny from females testing positive for WN viral RNA were used in subsequent experimentation. Within 24 h after hatching, empty egg rafts from each female were placed into cryovials and frozen at −80°C for later testing. All first-instar larvae kept separate by family were partitioned into two groups with at least 25 families per group. Group one larvae were immediately removed from oviposition vials, strained using vacuum filtration, and frozen at −80°C for later testing. Group two larvae were reared under autumnal conditions of 20°C and a photoperiod of 12:12 (L:D) h, in clear plastic 450-ml containers and fed a 1:1 mix of rabbit chow and fish food as needed. Emerging adults were held up to 5 d postemergence, anesthetized with TEA, separated into groups of 10 according to sex, and frozen in cryovials at −80°C until later testing.

Diagnostics. All viral assays were done under BSL-3 conditions at the CVEC laboratory. For mosquito adults, cryovials contained two 5-mm glass beads and 1.0 ml of mosquito diluent (Dulbecco's modified Eagle's medium containing 5% penicillin, 0.4% amphotericin, and 20% fetal bovine serum [FBS]). Egg raft and larval cryovials contained two 5-mm and four 1-mm glass beads and 1.0 ml of mosquito diluent. Samples were homogenized by mixer mill (MM300, Retsch, Haan, Germany), and total RNA was extracted using a MagMAX Express-96 system following the manufacturer's protocols (Invitrogen, Carlsbad, CA). Each extraction plate contained a positive control generated from cultured virus of known titer and at least two negative controls (mosquito diluent). Samples were tested for the presence and quantity of WN viral RNA by TaqMan One-Step (Invitrogen) and SensiFAST Probe Lo-ROX (Bioline USA Inc., Taunton, MA) real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using an ABI ViiA 7 real-time PCR platform. Initial screening of samples was done using the TaqMan assay per manufacturer protocols in singleplex (one primer/probe set). Plates containing positive samples were retested using the SensiFAST Probe Lo-ROX assay per manufacturer protocols in duplex (two primer/probe sets). The singleplex reaction was specific for the envelope region of the viral genome (WN1) (Lanciotti et al. 2000): 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. The duplex reaction included primers/probes for WN1 and the nonstructural (NS1) region of the viral genome (WN2) (Shi and Kramer 2003): 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. Samples with a cycle threshold (Ct) score <40 were considered positive, and Ct scores presented as the mean of screening and confirmation assays. All plates contained a standard curve generated from cultured virus of known titer (PFUs per ml) and negative water controls.

Bloodmeal titer was calculated by plaque assay (Kramer et al. 2002) 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% CO_2 for 1 h 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 h after absorption a second overlay (nutrient media, 1% agarose, 3% sodium bicarbonate, and 3% neutral red) was applied. After 72 h, plaques were counted, and virus concentration was calculated as PFU per ml.

Larvae from field-collected females that were positive for WN viral RNA also were assayed for infectious virus by Vero cell plaque assay before and after blind passage in C6/36 *Aedes albopictus* (Skuse) cells. For C6/36 infections, 200 μl from each sample was added to a 25-cm^2 tissue culture flask containing 70% confluent C6/36 cells from which all but 2.0 ml of tissue culture media (Dulbecco's modified Eagle's medium containing 10% FBS and 0.2% Primocin TM, InvivoGen, San Diego, CA) had been removed. An additional 2.0 ml of culture media was added after an absorption period of 1 h at 28°C and 5% CO_2. After 7 d, the tissue culture media were collected and tested for the presence of infectious virus by Vero cell plaque assay.

Statistics. The vertical transmission rate (VTR) is defined as the percentage of infected females that transmitted WNV to their egg raft or progeny, regardless of the proportion of progeny in each family infected (Turell 1988). 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) was calculated as the minimum number of mosquitoes infected with WNV per 1,000 offspring by using the bias-corrected maximum likelihood estimate (Biggerstaff 2003) with 95% confidence intervals (CL). Mosquitoes of each sex were tested in pools of

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Table 1. Field vertical transmission results for *Cx. quinquefasciatus*, *Cx. pipiens*, and *Cx. tarsalis* collected from WNV transmission foci in California

Location	Date	*Culex* species	Females laid eggs	Females testing positive (IR[a])	Larvae testing positive (VTR[b])	Eggs testing positive (%[c])
Los Angeles	24 Aug.	*quinquefasciatus*	56	3 (54)	1 (33)	NA[d]
	25 Aug.		12	0 (0)	0 (0)	NA
	31 Aug.		98	1 (10)	0 (0)	NA
		Total	166	4 (24)	1 (25)	NA
Kern	19 Aug.	*quinquefasciatus*	63	3 (48)	0 (0)	NA
	23 Aug.		214	10 (47)	3 (30)	NA
		Total	277	13 (47)	3 (23)	NA
Kern	6 Sept.	*tarsalis*	201	7 (35)	1 (14)	NA
		Total	201	7 (35)	1 (14)	NA
Sacramento[f]	19 Aug.	*pipiens*	105	7 (66)	3 (43)	4 (57)
	14 Sept.		172	3 (17)	1[e] (33)	1 (33)
	3 Oct.		13	0 (0)	0 (0)	0 (0)
		Total	290	10 (34)	4 (40)	5 (50)
		Overall total	934	34 (36)	9 (26)	NA

[a] Infection rate: actual number of mosquitoes infected with WNV per 1,000 offspring.
[b] Vertical transmission rate: percentage of females transmitting virus to their progeny, regardless of the proportion of progeny infected.
[c] Calculated by dividing the number of eggs testing positive by the number of females testing positive.
[d] NA, not available.
[e] Virus isolation in Vero cell culture.
[f] Data presented previously in Fetcher-Leggett et al. (2012) and included here for comparison.

≤10 adults. The Pearson chi-square test (Minitab 15, Minitab Statistical Software, Inc., State College, PA) was used to test for significant differences in the VTR between larval and adult progeny and between females infected with high (Ct < 24) and low (Ct > 28) quantities of WN viral RNA.

Results

Field Investigations. Field vertical transmission results for *Culex* females collected in three California counties are summarized in Table 1. Overall, 934 of the 945 (99%) field-collected females laid eggs that hatched, of which 34 tested positive for WN viral RNA by qRT-PCR, yielding an infection rate of 36 per 1,000. Of these, nine families of first-instar larvae tested positive, giving an overall field VTR of 26% (9/34). One infected female from Sacramento County did not lay eggs, bringing the overall female field infection rate to 37 per 1,000 (35/945).

Vertical transmission rates varied among species and locations. In Los Angeles County, trapping of gravid *Cx. quinquefasciatus* females occurred on 24, 25, and 31 August 2011. Gravid traps were set for one night each at three of four trap sites and for two trap nights at the remaining site. WN viral RNA was recovered from females at three of the four trap sites. Overall, 166 gravid females laid eggs that hatched, of which four females tested positive for WN viral RNA yielding an overall infection rate 24 per 1,000. Of these, one female vertically transmitted to larval progeny, giving a VTR of 25% (1/4).

In Kern County, *Cx. quinquefasciatus* females were trapped on 19 and 23 August, whereas *Cx. tarsalis* females were collected on 6 September 2011. Gravid traps were set for one night each at two of three trap sites and for two trap nights at the remaining site; CO_2 traps were set for one trap night each at three sites. West Nile viral RNA was isolated from females trapped at all sites, except for *Cx. tarsalis* at one site. Thirteen of the 277 gravid *Cx. quinquefasciatus* females that produced progeny tested positive for WN viral RNA, yielding an infection rate of 47 per 1,000. Of these, three families of first-instar larvae tested positive, giving a VTR of 23% (3/13). Seven of 201 *Cx. tarsalis* females tested positive, yielding an infection rate of 35 per 1,000. Of these, only one family of first-instar larvae tested positive giving a VTR of 14% (1/7).

In Sacramento County, trapping of *Cx. pipiens* complex females occurred on 19 August, 14 September, and 3 October 2011. Gravid traps were set for one night each at four traps sites and WN viral RNA was detected from females collected at all sites. Two hundred ninety-seven gravid females were captured, and of these 11 had WN viral RNA (infection rate, 37/1,000). However, only 10 of 290 females that laid viable eggs were infected, yielding an infection rate of 34/1,000. Of these, four families of first-instar larvae tested positive, giving a VTR of 40% (4/10). Empty egg rafts deposited by infected females also were tested for WN viral RNA and vertical transmission to eggs was 50% (5/10).

When the amount of viral RNA within field-collected mothers from all sites was examined, only those females that had a mean Ct score ≤20, indicating a virus body titer $\geq 10^5$ PFU/ml (based on standards on each sample plate), vertically transmitted WNV to their first-instar progeny. However, not all females with high-titered infections vertically transmitted to eggs or larvae. The single female from Sacramento County that vertically transmitted to eggs and not

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Table 2. Experimental vertical transmission of WN viral RNA to larval and adult progeny of infected *Cx. pipiens* females

Mean Ct score of infected females (viral titer)[a]	F₁ progeny tested for WN viral RNA					
	Larvae			Adults		
	No. families tested	No. + egg rafts/no. tested (%)	No. + families/no. tested (VTR[b])	No. families tested	No. + egg rafts/N=no. tested (%)	No. + families/no. tested (VTR[b])
< 24 (>10^4)	23	18/23 (78)	21/23 (91)	24	18/24 (75)	2/24 (8.3)
> 28 (<10^3)	4	0/4 (0)	2/4 (50)	1	0/1 (0)	0/1 (0)
Total	27	18/27 (67)	23/27 (85)	25	18/25 (72)	2/25 (8)

[a] Mean qRT-PCR Ct (cycle threshold) of initially infected females. West Nile viral titer expressed as plaque forming units (PFU) per milliliter.
[b] Vertical transmission rate: percentage of females transmitting virus to their progeny, regardless of the proportion of progeny infected.

larvae had a mean Ct score of 16 (>10^6 PFU/ml). All larval samples from Kern and Los Angeles counties and three of four larval samples from Sacramento County that tested positive by WN1 were confirmed by WN2 probe/primers. However, infectious virus was isolated from only one of nine larval pools before and after passage in C6/36 cells followed by Vero-cell plaque assay (Table 1).

Laboratory Experiments. The bloodmeal fed to *Cx. pipiens* females contained an estimated WNV titer of $10^{8.2 \pm 0.07}$ PFU/ml. The mean titer of the undigested blood within 20 engorged females was $10^{5.5 \pm 0.19}$ PFU/ml; differences in concentrations reflected the small volume of blood imbibed by these engorged females approximately 3–5 μl (Klowden and Lea 1978). Of the 200 females that fed on infectious blood, 76 survived 15 d post infection. Of these, 53 laid viable egg rafts, of which 52 tested positive for WN viral RNA by qRT-PCR. Of these positive females, 90.4% (47/52) had Ct scores <24, indicating a high virus body titer (>10^4 PFU/ml). Families from 28 and 25 infected females were used to evaluate vertical transmission to first-instar larvae and adult progeny, respectively.

Twenty-three of the 28 infected females vertically transmitted WNV to their first-instar larval progeny, giving an overall VTR of 85% (Table 2). In contrast, only two of 25 infected females transmitted WNV vertically and transstadially to their adult progeny, giving an overall VTR of 8.0%; in one of these families, WN viral RNA was detected in four pools of 10 males and one pool of 10 females, indicating a high infection rate. In the family from the second positive female, only one pool of four females contained WN viral RNA. Overall, vertical transmission rates were significantly higher when measured for first-instar larvae compared with adults ($\chi^2 = 30.98$, df = 1, $P < 0.0001$). Vertical transmission rates were 91.0 and 8.3% in first-instar larval and adult progeny, respectively, from females with high body titers (>10^4 PFU/ml) of WNV (Table 2). In contrast, infected females with low body titers (<10^3 PFU/ml) vertically transmitted to only 50% of larval and no adult progeny. However, vertical infection rates were not significantly different between females with high or low quantities of WN viral RNA ($\chi^2 = 0.14$, df = 1, $P = 0.70$). Rearing success was excellent, with intrafamily mortality ranging from 0 to 15 dead immatures (mean = 4.5; SD, ±5.16) per family.

The distribution of mean Ct scores among infected females was used to evaluate the titer of WNV in the parental females necessary for virus transmission to adult progeny (Table 3). The MFIR was 7.1 (95% CL, 2.92–14.69) infected adult progeny per 1,000 adult progeny tested ($n = 865$) from infected females, with a mean Ct score range of 16–20 (10^6–10^5 PFU/ml). Interestingly, no females ($n = 9$) with mean Ct scores <16 (>10^6 PFU/ml) vertically transmitted WNV to adult progeny. Virus also could not be detected in adult progeny from parent females with Ct scores >20 (<10^5 PFU/ml). The overall MFIR was 3.5 (95% CL, 1.42–7.18) infected adult progeny per 1,000 tested ($n = 1,750$). Because first-instar larvae were not enumerated by family, the MFIR could not be calculated.

The percentage of females passing virus vertically also was estimated by testing egg rafts. Interestingly, ovipositing egg rafts positive for WNV RNA did not assure that the F_1 progeny would become infected. Among females transmitting to larvae, seven had neg-

Table 3. Minimum filial infection rates of male and female adult progeny from infected *Cx. pipiens* females with varying quantities of WN viral RNA

Mean Ct score range of infected females (viral titer)[a]	No. of females	No. positive male pools/no. pools tested	No. positive female pools/no. pools tested	Total positive pools/total pools tested (total no. of mosquitoes)	MFIR[b]
< 16 (>10^6)	9	0/28	0/31	0/59 (516)	<1.9/1,000
16–20 (10^6–10^5)	11	4/50	2/49	6/99 (865)	7.1/1,000
20–24 (10^5–10^4)	4	0/12	0/12	0/24 (187)	<5.3/1,000
> 28 (<10^3)	1	0/11	0/9	0/20 (182)	<5.5/1,000
Total	25	4/101	2/101	6/202 (1,750)	3.5/1,000

[a] Mean qRT-PCR Ct (cycle threshold) of initially infected females. WN viral titer expressed as PFUs per milliliter.
[b] Minimum filial infection rate: minimum number of mosquitoes infected with WN virus per 1,000 offspring calculated using the bias-corrected MLE.

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ative egg rafts and positive larvae and one had a positive egg raft and negative larvae. A smaller proportion of females laid positive egg rafts (67%) than had positive larval progeny (85%) (Table 2). In contrast, among those transmitting to reared progeny, a similar percentage vertically transmitted virus to their egg rafts (72%) as did those tested as first instars; however, few pools of adult progeny were positive (8%). The two females that vertically transmitted to adult progeny had positive egg rafts. All egg rafts from infected females with a mean body Ct scores >28 were negative.

Discussion

Overwintering *Culex* females that were vertically infected during late summer and fall may serve as reservoirs of WNV that subsequently initiate transmission the following spring. However, there is a paucity of data on the frequency of vertical transmission to the overwintering generation in field populations. The current study provided evidence that vertical transmission of WNV occurs in natural populations of *Culex* mosquitoes during late summer and fall and may be a potential mechanism inserting virus into the overwintering generation. In addition, we experimentally compared the VTR by measuring WN viral RNA in first-instar larvae and adult progeny, and we demonstrated that a considerable number of viral infections may be lost transstadially during larval development to the adult stage. Because our larval mortality was very low, virus likely was lost during molting, metamorphosis, or both, and was not due to the death of infected individuals.

Field studies on three *Culex* species detected frequent vertical transmission of WNV from infected females to first-instar progeny at late season foci of transmission in three California counties. Our field estimate of an overall VTR of 26% was similar to a previous laboratory study (Reisen et al. 2006a) that showed about one in four *Cx. tarsalis* females were able to transmit WNV transgenerationally. However, our rate is higher than a previous field estimate that found a VTR of 10% in *Cx. pipiens* (Anderson and Main 2006). In the current study, field vertical infection rates in *Cx. tarsalis* were relatively low (14%) compared with members of the *Cx. pipiens* complex (25, 23, and 40%). One explanation for these data is that *Cx. tarsalis* were collected using CO_2-baited traps, and such traps may collect a higher proportion of younger females than gravid traps used for collecting *Cx. pipiens* complex females. In addition, *Cx. tarsalis* females were collected in September when transmission rates appeared to decline in concurrently collected *Cx. pipiens.* No females positive for WNV were collected in October, although low sample size due to declining abundance may have been a factor.

As expected, our data showed that vertical transmission was only detected in the progeny of females with elevated virus titers. Previously, the frequency of vertical transmission was found to increases with female gonotrophic age (Anderson et al. 2008); therefore, field rates were expected to increase during late season, when older *Culex* females and high temperatures for virus replication were present. In addition, dissemination of WNV to the ovaries in *Cx. quinquefasciatus* females was found to occur after 14 d postinfection (Girard et al. 2004). In the laboratory, we held mosquitoes up to 15 d postinfection at 26°C to allow virus dissemination to the ovaries.

Our experimental VTR of 8% measured in adult progeny was similar to a previous laboratory study using *Cx. pipiens* that reported a VTR of 5% (Anderson et al. 2008). In contrast, our vertical transmission rate to first-instar larvae was 85%, the highest yet reported for a flavivirus. It was unclear why WNV was detected at such high rates in first-instar larval progeny compared with adult progeny. Because larval and pupal mortality was low in the current experiment, virus was apparently degraded or lost transstadially during larval development, molting or pupal metamorphosis. Previous studies have investigated flavivirus vertical transmission to third- and fourth-instar larvae and pupae (Rosen et al. 1978, Rosen et al. 1983, Hardy et al. 1984, Nayar et al. 1986), including WNV (Baqar et al. 1993), and found higher rates of vertical transmission to immatures compared with adults. Transstadial transmission of flaviviruses from infected larval to adult progeny in *Culex* females seems to be inefficient. Hardy et al. (1984) suggested that the virus may be inactivated during larval-pupal metamorphosis, pupal-adult metamorphosis, or both. However, in one instance the MFIR for one field strain of *Cx. tarsalis* infected with St. Louis encephalitis virus (family *Flaviviridae,* genus *Flavivirus*) was comparable in F_1 adults compared with F_1 larvae (Hardy et al. 1984).

For our laboratory investigations, larvae were reared at 20°C and a photoperiod of 12:12 (L:D) h, simulating the photoperiod and water temperature during early autumn in California. Previous studies have found the transstadial transmission of flaviviruses was enhanced at cooler rearing temperatures (Hardy et al. 1980, 9184; Nayar et al. 1986). Filial infection rates for WNV among *Culex tritaeniorhynchus* Giles pupae reared at 20°C were higher than those reared at 26°C (Baqar et al. 1993). Cool temperature during autumn therefore may enhance the ability of flaviviruses to be transstadially transmitted to overwintering adult female populations.

Minimum filial infection rates of WNV reported from field and laboratory studies have varied. In laboratory investigations, MFIRs in *Culex* as high as 8.1WNV infected progeny per 1,000 tested (Reisen et al. 2006a) and as low as 0.04 WNV infected progeny per 1,000 tested (Anderson et al. 2012) have been reported. Our overall rate of 3.5 WNV infected progeny per 1,000 tested is similar to both field (Anderson and Main 2006) and laboratory (Goddard et al. 2003) studies done with *Cx. pipiens* and *Cx. quinquefasciatus* females, respectively. In general, MFIRs for flaviviruses have been shown to be significantly lower than those reported for bunyaviruses, with rates up to 500 infected progeny per 1,000 tested (Miller et al. 1977). Bunyaviruses infect the germ tissues of developing

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follicles in the ovary (Tesh and Cornet 1981), whereas flaviviruses apparently infect the fully formed egg during deposition (Rosen 1988).

In Sacramento County, the field VTR was 50% in empty egg rafts and 40% in first-instar larvae. We also saw higher VTR to egg rafts (72%) than adult progeny (8%) in experimental studies. One explanation is larvae may acquire the virus by feeding on infected egg rafts after eclosion. However, Rosen et al. (1978) found only one of 64 *Ae. albopictus* larvae became infected when eggs were hatched in media containing 10^6 PFU/ml of Japanese encephalitis virus (family *Flaviviridae*, genus *Flavivirus*, JEV). This study also isolated JEV in F_1 generation *Aedes* mosquitoes that were reared from eggs that had been dried and kept at room temperature for up to 2 mo, demonstrating that JEV was actually inside the mosquito egg. Alternatively, virus dissemination to the accessory glands of infected females may account for the high rates of vertical transmission to egg rafts seen in the current study. The virus may persist for a variable duration in the accessory gland fluid that attaches the eggs during oviposition.

Because the efficiency of vertical transmission can be influenced by viral strain, rearing temperature, mosquito species and strains, and number of gonotrophic cycles completed by infected females (Hayes et al. 1984; Baqar et al. 1993; Anderson et al. 2008, 2012), testing first-instar larvae for WNV from locations with active horizontal transmission in late summer and fall also may identify areas with high rates of vertical transmission (Fechter-Leggett et al. 2012). This could be used as a surveillance tool to focus mosquito control efforts during summer and before virus amplification in spring. Our data indicate that infected mosquitoes collected from foci of virus activity late in the season are capable of passing WNV into progeny destined for overwintering. Future studies are needed to explain how WNV is vertically transmitted, if larvae can become infected by feeding on infected eggs, and why virus seemed to be frequently degraded during metamorphosis.

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