Overwintering of West Nile Virus in Southern California

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ABSTRACT West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) invaded southern California during 2003, successfully overwintered, amplified to epidemic levels, and then dispersed to every county in the state. Although surveillance programs successfully tracked and measured these events, mechanisms that allowed the efficient overwintering and subsequent amplification of WNV have not been elucidated. Our current research provided evidence for three mechanisms whereby WNV may have persisted in southern California during the winters of 2003–2004 and 2004–2005: 1) continued enzootic transmission, 2) vertical transmission by *Culex* mosquitoes, and 3) chronic infection in birds. WNV was detected in 140 dead birds comprising 32 species, including 60 dead American crows, thereby verifying transmission during the November–March winter period. Dead American crows provide evidence of recent transmission because this species always succumbs rapidly after infection. However, WNV RNA was not detected concurrently in 43,043 reproductively active female mosquitoes comprising 11 species and tested in 1,258 pools or antibody in sera from 190 sentinel chickens maintained in 19 flocks. Although efficient vertical transmission by WNV was demonstrated experimentally for *Culex tarsalis* Coquillett infected per os, 369 females collected diapausing in Kern County and tested in 32 pools were negative for WNV. Vertical transmission was detected in *Culex pipiens quinquefasciatus* Say adults reared from field-collected immatures collected from Kern County and Los Angeles during the summer transmission period. Chronic infection was detected by finding WNV RNA in 34 of 82 birds that were inoculated with WNV experimentally, held for >6 wk after infection, and then necropsied. Frequent detection of WNV RNA in kidney tissue in experimentally infected birds >6 wk postinfection may explain, in part, the repeated detection of WNV RNA in dead birds recovered during winter, especially in species such as mourning doves that typically do not die after experimental infection. In summary, our study provides limited evidence to support multiple modes of WNV persistence in southern California. Continued transmission and vertical transmission by *Culex p. quinquefasciatus* Say seem likely candidates for further study.

KEY WORDS West Nile virus, overwintering, southern California, *Culex tarsalis*, *Culex pipiens quinquefasciatus*

WEST NILE VIRUS (family *Flaviviridae*, genus *Flavivirus*, WNV) invaded southern California during summer 2003 (Reisen et al. 2004b), overwintered successfully, amplified to epidemic levels during 2004 (Hom et al. 2005), and then invaded the Central Valley leading to an outbreak with high case incidence in Bakersfield (Takahashi et al. 2005). Isolates of the WNV strain invading California were relatively similar genetically and in virulence to the original NY99 isolate (Davis et al. 2005). Our on-going research attempts to elucidate mechanisms that enable the effective persistence and amplification of this invading virus in California.

Among the potential mechanisms responsible for the persistence of arboviruses in temperature biomes (Reeves 1987, 1990; Rosen 1987a), three seem most plausible to explain the persistence of WNV in southern California and are supported by limited field data: 1) continued transmission, 2) vertical transmission to females destined for reproductive diapause that may carry WNV through winter and then transmit virus to avian hosts during spring, and 3) chronic infection of WNV in avian hosts that relapse during spring. In

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California, the period during which the primary encephalitis vector, *Culex tarsalis* Coquillett, undergoes reproductive diapause historically has delineated the "winter period" and heralded the interruption of arbovirus transmission during fall (Bellamy and Reeves 1963, Reeves 1990). Even in mild southeastern California, *Cx. tarsalis* females emerging after mid-October remain in reproductive diapause until after the winter solstice in late December (Reisen et al. 1995b). Diapause termination seems to be temperature related and is delayed as a function of latitude, being several weeks later in northern Kern County than in southern Coachella Valley (Bellamy and Reeves 1963; Reisen et al. 1986b, 1995b).

WNV enzootic activity during winter has been detected elsewhere at southern latitudes by the detection of virus in dead birds and *Culex pipiens quinquefasciatus* Say from southern Texas (Tesh et al. 2004) and by seroconversion of sentinel chickens in Florida (Shaman et al. 2005). Detection of virus within dead or viremic avian hosts and reproductively active mosquito hosts as well as the seroconversion of sentinels support the concept of continued transmission. Therefore, one objective of our study was to detect virus activity during winter in California by testing dead birds reported by the public, pools of reproductively active mosquitoes collected while host seeking or attempting to oviposit in gravid traps, and sera from sentinel chickens maintained and bled during winter.

WNV has been recovered from diapausing *Culex pipiens pipiens* L. in New York (Nasci et al. 2001), New Jersey (Farajollahi et al. 2005), and Pennsylvania (Bugbee and Forte 2004). However, attempts to recover virus from overwintering *Culex* mosquitoes after the Colorado epidemic were unsuccessful (Moore et al. 2005), questioning whether WNV persists in diapausing females of other species and/or strains of *Culex.* Because females destined for diapause will not blood feed (Mitchell 1981, Mitchell and Briegel 1989, Spielman 2001), infection presumably occurs by vertical transmission (Rosen 1987a). Based on laboratory proof of principle experiments using females infected by intrathoracic inoculation (IT) and progeny reared en masse, vertical passage of the NY99 strain of WNV has been demonstrated for several North American *Culex* species (Dohm et al. 2002, Goddard et al. 2003). However, these experimental protocols did not determine whether 1) females could transmit virus vertically after being infected per os or 2) what proportion of infected female parents transmit virus vertically. Previously, vertical passage of WNV by females infected per os was demonstrated experimentally for *Culex tritaeniorhynchus* Giles from Pakistan by using the Egypt 101 strain of WNV (Baqar et al. 1993), and WNV was isolated from field-collected *Culex univittattus* Theobald males from Kenya (Miller et al. 2000). Therefore, a second objective of our research was to determine whether the NY99 strain of WNV could be passaged vertically by *Cx. tarsalis* females infected by feeding on a donor avian host and what proportion of these infected females were capable of vertical transmission. In addition, we tested adults emerging from wild-caught immatures and field-collected males during summer and adults of both sexes collected during winter to determine whether vertical passage could be detected in field populations in California. The frequent recovery of infected males during experimental vertical transmission experiments (Goddard et al. 2003) and the recovery of virus from naturally infected males in Kenya (Miller et al. 2000) led us to also investigate whether these male infections were a dead end for the virus or if these infected males could transmit virus venereally.

Long-term infections of avian hosts with arboviruses have been described after experimental infection (Reeves et al. 1958, Semenov et al. 1973, Reisen et al. 2003c) and similar infections followed by vernal relapse were considered to be responsible for annual spring emergence of eastern equine encephalomyelitis virus (family *Togaviridae,* genus *Alphavirus,* EEEV) in New Jersey (Crans et al. 1994). Because several dead birds collected during winter have been confirmed to be positive for WNV RNA and/or infectious virus (Anderson et al. 1999; Hom et al. 2004, 2005; Tesh et al. 2004), we investigated the frequency of WNV chronic infections among experimentally infected birds by using the same procedures and criteria established previously for other encephalitis viruses (Reisen et al. 2003c). Although previous studies showed that birds were protected by immunity acquired from the initial infection and were not infectious (Reisen et al. 2003a), birds dying during winter from other causes could contain WNV RNA-positive organs and therefore be reported as positives by dead bird surveillance programs, even though these birds may not represent recent transmission events. Our third objective was to determine the frequency of chronic infections among different bird species after experimental inoculation with WNV.

Methods and Materials

Field Areas. All field collections during winters 2003–2004 and 2004–2005 were done in southern California in collaboration with the Coachella Valley Mosquito and Vector Control District (MVCD) in Riverside County, the Greater Los Angeles County Vector Control District (VCD) in Los Angeles County, and the Kern MVCD in Kern County. Descriptions of study areas within these districts, the mosquito fauna, and patterns of endemic arbovirus transmission have been published previously (Reisen et al. 1992b, c, 1995a, 2002b). Daily ambient temperatures from weather stations in each of these areas were downloaded from the University of California Statewide IPM Program Web site (http://www.ipm.ucdavis.edu/).

Continued Enzootic Transmission. We attempted to document continued WNV transmission during winter by detecting infection in dead birds reported by the public or found by field personnel and in reproductively active mosquitoes, or by seroconversion of sentinel chickens. Dead birds reported by the public to the Dead Bird Hot Line at the California Department

of Health Services and confirmed to be intact and dying within the previous 24–48 h (McCaughey et al. 2003) were necropsied by the California Animal Health and Food Safety Laboratory and tissue samples sent to Center for Vectorborne Diseases laboratory (CVEC) at University of California, Davis, for testing. Brain, heart, lung, and kidney tissue pools were tested during 2003–2004; however, during 2004–2005 testing was limited to oral swabs from American crows and kidney snips or swabs from remaining species to enhance throughput. During 2003–2004, sampling emphasized corvid and raptor species, whereas during 2004–2005 all species were accepted for testing.

Mosquitoes collected in gravid traps (Cummings 1992) or host seeking at dry ice-baited traps (Newhouse et al. 1966) operated during November–March were pooled into lots of ≤50 females and shipped to CVEV for testing.

Flocks of 10 sentinel hens were deployed in Imperial Valley (three flocks), Coachella Valley (five flocks), Los Angeles (five flocks), and Kern County (six flocks) and were bled at biweekly or monthly intervals by using a lancet prick of the comb (Reisen et al. 1993a) or by jugular puncture. Blood samples on filter paper strips were stored at room temperature, whereas whole sera were stored at −80°C. Whole serum samples taken in March were tested by enzyme immunoassay to detect seroconversions during the winter (Chiles and Reisen 1998). All samples from end of winter positive chickens could be tested retrospectively to determine when these seroconversions occurred.

Vertical Infection. The frequency of vertical infection was studied in the laboratory and the field.

Laboratory Experiments. Experimental infections used the NY99 strain of WNV isolated originally from a Chilean flamingo at the Bronx Zoo (strain 35211 AAF 9/23/99). Stock virus was at Vero cell passage 2 or 3 and had a titer of 7.9–8.6 $\log_{10}$ plaque forming units (PFU) per milliliter. In experiment 1, females from the Kern National Wildlife Refuge (KNWR) laboratory colony of *Cx. tarsalis* established from Kern County during 2002 were infected orally by feeding on a viremic House finch 2 d postinfection (dpi) (viremia, 5.5 $\log_{10}$PFU/ml) or by IT inoculation with ≈5 $\log_{10}$ PFU of WNV. Females infected by IT inoculation were held on 10% sucrose for 10 d at 26°C and then blood fed on an uninfected chick. Females infected per os were maintained concurrently. When gravid, females were placed individually into vials for oviposition, and the resulting families were reared individually in enameled trays on a diet of finely ground alfalfa pellets and fish food. Teneral adults were maintained for >3 d at room temperature (22–24°C) on 10% sucrose until separated by sex and enumerated into pools of ≤25 individuals each.

Because vertical infection rates in experiment 1 were lower than expected and because different strains of *Cx. tarsalis* reportedly vary in their ability to transmit WNV vertically (Goddard et al. 2003), we repeated the oral infection portion of experiment 1 by using a colony of *Cx. tarsalis* established from Yolo County, CA, during 2003. In experiment 2, Yolo females were infected per os by feeding on a white-crowned sparrow (2 dpi) with a viremia of 7.2 $\log_{10}$ PFU/ml and then were maintained, reared, and pooled as described in experiment 1.

In previously published studies (Goddard et al. 2003), male progeny frequently were found to be infected vertically. Therefore, we conducted experiments to determine whether infected males were dead end hosts or could transmit WNV venereally to females during mating. In experiment 3, 10 males from the Bakersfield (BFS) laboratory colony of *Cx. tarsalis* were infected by IT inoculation with ≈5 $\log_{10}$ PFU of WNV, held for 7 d, and then individually offered three virgin females overnight. When dissected the next day, females were not inseminated and were discarded. We then attempted to force-mate (Baker 1964) each of the 10 surviving males to one to three anesthetized unfed virgin females. Males were frozen at −80°C immediately after mating for later virus testing, whereas females were held 24 h before freezing at −80°C.

Because two of three inseminated females from experiment three were positive for WNV, indicating that venereal transmission had occurred, we investigated the ability of venereally infected females to transmit virus vertically. In experiment 4, 50 3- to 4-d-old males from the KNWR colony were inoculated IT with ≈5 $\log_{10}$ PFU of WNV, maintained on 10% sucrose at 26°C for 7 d, and then used in attempted force-matings with freshly blood-fed virgin females; males successfully mating were frozen for virus testing to ensure infection status. Females were held for 3 d until gravid and then placed in individual vials for oviposition. Female parents were frozen individually after oviposition and then tested for viral infection. Progeny from infected females were reared individually as described in experiment 1.

Field Investigations. During summer when WNV enzootic transmission was detected by positive mosquito pools or dead birds or by sentinel chicken seroconversions (Hom et al. 2004, 2005), we attempted to detect vertical infection by testing males collected in gravid female traps (Cummings 1992) in Los Angeles or in suction traps (Lothrop et al. 2001) positioned near nectar sources in Coachella Valley. In addition, field-collected immature mosquitoes were reared under ambient (Los Angeles) or insectary conditions of 24°C and a photoperiod of 18:6 (L:D) h (Coachella Valley, Kern County), maintained for >3 d postemergence, separated by sex, and then pooled into lots of ≤50 each.

From October to December 2004, adult *Culex* resting under bridges, in outdoor rest rooms and inside abandoned buildings throughout Kern County were collected by mechanical aspirator, enumerated to sex and species, and then pooled in lots of ≤50 each for later testing.

Chronic Infection. Birds were infected by subcutaneous inoculation with ≈3,000 PFU of WNV, and viremia was measured for 6–8 d postinoculation (Reisen et al. 2005). Surviving birds were maintained

Table 1. Dead birds reported by the public, necropsied, and tested for WNV RNA during the winter periods of 2003–2004 and 2004–2005 at study areas in southern California and the rest of the state

Common name	Scientific name	2003–2004				2004–2005			
		Nov.–Dec.		Jan.–Mar.		Nov.–Dec.		Jan.–Mar.	
		Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
Southern study area									
American crow	*Corvus brachyrhynchos*	14	7	3	0	0		5	0
Total		18	7	6	0	8	0	35	1[a]
All of California									
American crow	*Corvus brachyrhynchos*	153	34	161	2	84	12	174	12
Pine siskin	*Carduelis pinus*	0		0		1	0	88	7
House sparrow	*Passer domestica*	15	0	0		39	4	173	6
Lesser goldfinch	*Carduelis psaltria*	3	0	0		6	2	60	4
Yellow-rumped warbler	*Dendroica coronata*	10	0	0		6	1	35	4
Barn owl	*Tylo alba*	2	0	0		0		9	3
Brewer's blackbird	*Euphagus cyanocephalus*	1	0	0		18	2	35	2
House finch	*Carpodacus mexicanus*	16	0	3	0	19	0	88	2
Western scrub-jay	*Aphelocoma coerulescens*	9	0	0		14	5	42	2
Yellow-billed magpie	*Pica nuttali*	0		0		8	2	13	2
Species with 1 positive		0		0		124	11	141	13
Species negative		104	0	15	0	180	0	487	0
Total		313	34	10	2	499	39	1,345	57

[a] House finch.

under BSL3 containment for >6 wk postinfection and then necropsied. Blood (sera), spleen, kidney, and lung tissues were removed and screened for WNV infection by reverse transcript-polymerase chain reaction (RT-PCR).

Diagnostics. Mosquitoes, dead birds, and necropsy tissues were tested for WNV RNA by RT-PCR, after RNA extraction by using a robotic ABI 6100 system (Shi and Kramer 2003). RNA within avian blood samples was extracted using the one-step RNeasy system (QIAGEN, Valencia, CA). Host-seeking and gravid trap-collected female mosquitoes were assayed using a TaqMan multiplex assay that included primers and probes for WEEV, SLEV, and WNV. Screening primers for WNV were generated from E sequences (Lanciotti et al. 2000) as follows: (forward) 5′-TCA GCG ATC TCT CCA CCA AAG-3, (reverse) 5′-GGG TCA GCA CGT TTG TCA TTG-3′, (probe) 6FAM-TGC CCG ACC ATG GGA GAA GCT-TAMRA. The remaining specimens were assayed for WNV RNA by a singleplex TaqMan assay in which WNV-specific screening primers and probes were used exclusively. Positive RT-PCR results were confirmed using a second primer set from the NS1 gene of WNV [(forward) 5′-GGC AGT TCT GGG TGA AGT CAA-3′, (reverse) 5′-CTC CGA TTG TGA TTG CCT CGT-3′, (probe) 6FAM-TGT ACG TGG CCT GAG ACG CAT ACC TTG T-TAMRA]. Some positive specimens also were tested for infectious virus using a plaque assay on Vero cell culture (Kramer et al. 2002). Some necropsy tissues from experimentally infected birds that were positive by RT-PCR were passed in C6/36 cell culture before plaque assay on Vero cells to enhance detection (Reisen et al. 2003c). End of winter sentinel chicken bloods were screened for IgG antibody by enzyme immunoassay by using a flaviviral antigen (Chiles and Reisen 1998).

Results

Continued Enzootic Transmission. In total, 67 dead wild birds comprising 20 species were tested from our study areas during winter, of which eight tested positive from two species. In addition, 2,167 birds comprising 151 species were tested for WNV RNA throughout California, of which 132 from 32 species tested positive (Table 1). American crows were most frequently positive ($n = 60$, 45% of total positives), followed by house sparrows ($n = 10$), western scrub-jays ($n = 7$), and pine siskins ($n = 7$). Presence of viral RNA within birds testing positive during the winter–spring period was confirmed by retesting by using a second primer set from the NS1 gene of WNV.

Winter transmission was not detected within our three study areas by seroconversion of sentinel chickens. None of the 190 hens deployed in 19 flocks during winters 2003–2004 or 2004–2005 seroconverted to WNV. In addition, none of 43,043 reproductively active female mosquitoes comprising 11 species collected during the winter period and tested in 1,258 pools were positive for WNV (Table 2). Data were summarized for the November to March period, because WNV-positive pools were detected as late as October in Kern County and Los Angeles and as early as April in Coachella Valley. A single pool of *Cx. p. quinquefasciatus* females collected by gravid trap from Griffith Park near the Los Angeles Zoo on 19 November 2003 tested positive for SLEV RNA; this was the only SLEV positive pool recovered from Los Angeles during the 2003–2005 period and the last pool positive for SLEV in California to date.

For WNV to be transmitted during winter, temperature must exceed the thermal minimum required for virus replication within the mosquito host. For WNV, this temperature recently was estimated to be 14.3°C

Table 2. Number of pools and female mosquitoes collected by dry ice-baited or gravid female traps during the winters of 2003–2005 and 2004–2005 and tested for WNV RNA with negative results

Species	2003–2004		2004–2005	
	No. pools	No. tested	No. pools	No. tested
Coachella Valley				
Cx. tarsalis	155	6,549	202	7,384
Cx. p. quinquefasciatus	20	625	58	946
Cx. erythrothorax	46	2,061	1	50
Cs. inornata	24	288	52	879
Ae. vexans	9	153	8	248
Los Angeles				
Cx. tarsalis	10	183	4	104
Cx. p. quinquefasciatus	179[a]	5,969	153	4,352
Cx. stigmatosoma	14	150	4	51
Cx. erythrothorax	34	1,453	114	5,452
Cx. thriambus	1	20	1	50
Cs. incidens	16	274	11	314
Cs. inornata	3	51	1	4
Cs. particeps	1	22	0	0
An. hermsi	0	0	1	7
Kern County				
Cx. tarsalis	18	730	34	1,327
Cx. p. quinquefasciatus	2	16	4	23
Cx. erythrothorax	0		12	578
Ae. melanimon	36	1,695	26	1000
Cs. inornata	0		4	35
Total	568	20,239	690	22,804

[a] One pool positive for SLEV.

(Reisen et al. 2006). Figure 1 shows the average daily temperatures at our study sites during the January 2003 to March 2005 period. Mean temperatures during winter in Coachella Valley and upland Los Angeles (Monrovia) frequently were above the thermal minimum, whereas cool winter temperatures in coastal Los Angeles (Long Beach) and Kern County probably would not support virus replication within mosquitoes.

Vertical Transmission: Laboratory Experiments. Experiments 1 and 2 (Table 3) confirmed the ability of *Cx. tarsalis* to vertically transmit WNV. In experiment 1, none of the 92 pools comprised of 1,909 progeny from 29 females infected per os tested positive, whereas two of 104 pools comprised of 1,817 progeny from 39 females infected by IT inoculation were positive. The two positive pools were from different IT-inoculated female parents, indicating that two of 39 females (5%) transmitted virus vertically. The absence of transmission by females infected per os, questioned the ability of the NY99 strain of WNV to pass vertically in *Cx. tarsalis* after natural infection.

Experiment 2 evaluated the ability of the Yolo strain to pass WNV vertically after oral infection. All parent females developed a high virus titer (mean 6.6 $\log_{10}$ PFU per mosquito) and five of 21 (24%) passed virus vertically to their progeny (Table 3). Of 17 pools from the five females that vertically transmitted virus, nine were positive for WNV RNA, and virus was isolated on Vero cell culture with titers ranging from 4 to 6 $\log_{10}$ PFU per pool. Five of these nine positive pools consisted of males. Infection rates then were calculated for the progeny from all females to allow comparisons with values reported in the literature. Inspection of the 95% confidence interval estimated by a maximum likelihood procedure (Biggerstaff 2003) indicated that the overall filial vertical infection rate of 8.1 per 1000 for females infected orally was not significantly different from our previous estimate of 6.9 per 1000 for females collected host seeking in Yolo County and infected by IT inoculation (Goddard et al. 2003).

In experiment 3, all 10 males were infected after IT inoculation, and two of these were successfully force-mated to three females. These females were held overnight at 26°C and then frozen at −80°C for later testing. Two of these three force-mated females tested positive by RT-PCR, and virus was isolated from one of these two positive females (titer, 2.1 $\log_{10}$ PFU). This small experiment established that it was possible to transfer infectious WNV venereally through force-mating.

In experiment 4, all 18 males inoculated IT and successfully force-mated to freshly fed females that oviposited were infected (mean titer 6.8, range 5.6–7.3 $\log_{10}$ PFU/male mosquito). However, when female parents were tested for infection after oviposition 3–5 d later, only one had a low level infection (TaqMan Ct value = 25, plaque assay titer = 1.8 $\log_{10}$ PFU). Female progeny from this female were tested individually ($n = 30$), and the remaining adults in pools of 10 each ($n = 6$) with negative results. Collectively, data from experiments 3 and 4 indicated that although WNV can be transmitted venereally, it does not persist frequently in these females for the 3–5-d period required for the completion of the gonotrophic cycle. In addition, low titered virus within the single infected female was not passed with the sperm to the eggs during oviposition, because her progeny were not infected.

Vertical Transmission: Field Observations. In total, 11,322 adult *Culex* mosquitoes (three species) were tested for WNV vertical infection in 480 pools by singleplex RT-PCR (Table 4). WNV was detected only in *Cx. p. quinquefasciatus* collected during the summer. Positive pools consisted of 15 males collected from a gravid trap near Whittier, Los Angeles, 50 females reared from an egg raft oviposited in a gravid trap set at the Los Angeles Zoo, and 50 males reared from field-collected larvae collected near Lamont, Kern County. The presence of viral RNA was confirmed using a second primer set from the NS1 gene of WNV and by isolation of WNV from two of three pools by using Vero cell culture. These field data established the infrequent occurrence of vertical transmission within populations of *Cx. p. quinquefasciatus* during the summer transmission season. Evidence of vertical transmission was not detected for *Cx. tarsalis*, even though two pools of females collected concurrently with males in suction traps in Coachella Valley tested positive for WNV, indicating that WNV was active at the collection site when these males were sampled.

We were not able to detect WNV RNA in overwintering females that were collected during fall near sites in Kern County that supported epidemic transmission during summer 2004 (Takahashi et al. 2005). Although the numbers we tested were modest (Table 4), these

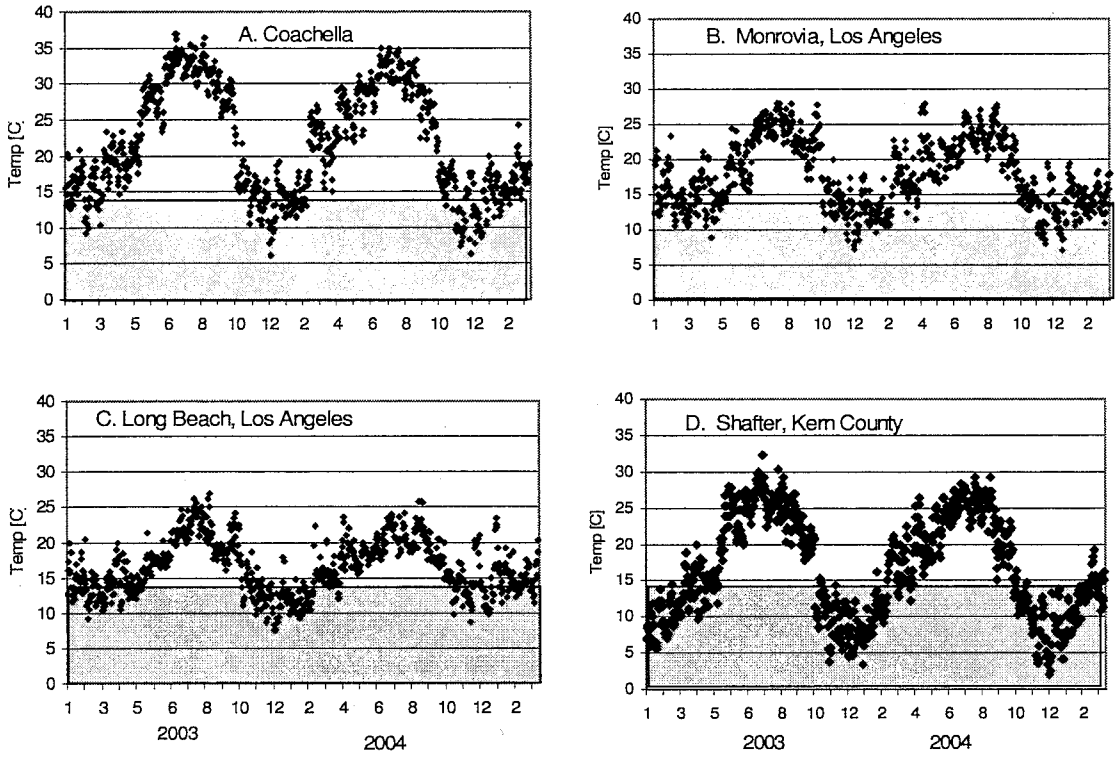

Fig. 1. Mean daily temperatures in the Coachella Valley, Riverside County, Monrovia, and Long Beach in Los Angles County, and Shafter, Kern County. Temperatures within the shaded areas were too low for WNV development within the mosquito host.

sample sizes required the efforts of a team of two to four collectors inspecting numerous shelters on 11 occasions. Efforts focused on overwintering refugia known to be productive for *Cx. tarsalis* during previous winter studies (Reisen et al. 1986b, c). Inspections were terminated in mid-December, because *Cx. tarsalis* typically terminates diapause in January (Bellamy and Reeves 1963), and females were not dissected to ensure nulliparity before testing.

Chronic Infection. Overall, 28 (34%) of 82 birds from six species had one or more organs positive for WNV RNA at necropsy >6 wk after experimental infection (Table 5). In addition, a single house sparrow had WNV RNA detected in its sera. Presence of viral RNA in all birds reported as positive was confirmed using a second primer set from the NS1 gene of WNV. House finches were most frequently chronically infected. Infectious virus was recovered by Vero cell plaque assay from four of six RT-PCR-positive house finches after passage of organ tissue extracts through C6/36 *Aedes albopictus* (Skuse) cell culture. Collectively, these data established that WNV RNA persists within the organs (spleen, kidney, and lung) of several species of birds but does not circulate frequently within the bloodstream. Recovery of WNV from four of six house finches after blind passage

Table 3. Vertical infection experiments with KNWR (exp. 1) and Yolo (exp. 2) colonies of *Cx. tarsalis* showing the number of families, pools, and total individuals tested and the number of positive pools

Colony	Infection method	
	Per os	IT
Exp. 1. KNWR colony		
No. of parent females	29	39
Male, pools	47	53
Male, total	936	916
Male, WNV positive	0	1
Female, pools	45	51
Female, total	973	901
Female, WNV positive	0	1
Exp. 2. Yolo colony		
No. females tested	21	
Parent females positive	5	
Progeny pools tested	17	
Progeny pools positive	9	
Progeny testing positive	340	
IR/1000 for positives	41.4	
IR (95% CL)	21.7–79.9	
Parent females negative	16	
Progeny pools negative	56	
Progeny testing negative	970	
IR overall	8.1	
IR (95% CL)	4.2–14.4	

Per os, infected by feeding on viremic bird; IT, infected by intrathoracic inoculation; IR, filial infection rate estimated by a maximum likelihood procedure (Biggerstaff 2003); CL, confidence limit.

Table 4. Attempted detection of vertical infection in three *Culex* species collected in Coachella Valley, Los Angeles, or Kern County during summer transmission seasons (June–Aug. 2003–2004) or fall diapause period (Oct.–Dec. 2004)

Location	Date	*Culex* species	Method	Sex	No. pools	Total
Coachella	June–Aug.	*tarsalis*	Suction trap	M	49	1,865
			Reared	M	31	1,198
			Reared	F	34	1,366
Los Angeles	June–Aug.	*quinquefasciatus*	Gravid traps	M	15	428[a]
			Reared	F	26	1,065[b]
			Reared	M	39	1,806
Kern	July–Sept.	*quinquefasciatus*	Gravid trap	M	8	102
			Reared	M	30	1,405[b]
			Reared	F	30	1,395
		stigmatosoma	Reared	M	10	390
			Reared	F	12	431
		tarsalis	Reared	M	11	388
			Reared	F	9	360
	Oct.–Dec.	*quinquefasciatus*	Resting	M	32	440
			Resting	F	73	858
		stigmatosoma	Resting	M	5	28
			Resting	F	22	209
		tarsalis	Resting	M	12	119
			Resting	F	32	369
				Total	480	11,322

[a] One pool positive by RT-PCR, virus isolation negative.
[b] One pool positive by RT-PCR and virus isolated in Vero cell culture.

indicated that the RNA was from infectious or at least potentially infectious virus.

Discussion

Our investigations on WNV winter persistence were conducted after the invasion of southern California (2003–2004) and the Central Valley (2004–2005) and epidemics in Los Angeles and Bakersfield (2004), and provided limited evidence supporting three very different persistence mechanisms: 1) continued transmission, 2) vertical transmission, and 3) chronic avian infection.

Continued Transmission. During both winters, continued WNV horizontal transmission was detected in our study areas in Los Angeles and Kern counties by the recovery of WNV RNA from seven and one dead bird, respectively. During summer 2004, there were 840 and 86 dead birds from these areas that tested positive for WNV, respectively. An additional, 124 positive birds were recorded during winter from the rest of California. The 73 birds positive during the November–December period may have been infected at the end of the fall transmission period, although WNV-positive host-seeking mosquitoes were not detected concurrently. Dead birds testing positive during the January–March period represented either recent or chronic infections. The two dead American crows detected in Los Angeles during January–March 2004 and the 12 American crows detected throughout California during January–March 2005 presumably died from recent infection, indicating low level virus transmission that was not detected by other surveillance methods. Dead American crows presumptively indicated a recent transmission event, because all American crows experimentally infected with the NY99 strain of WNV succumbed within 5 to 6 d postinfection (Komar et al. 2003, Brault et al. 2005). During the January–March 2005 period, 33 birds comprising 22 other species also tested positive. However, we could not resolve whether these infections were recent and the cause of death or whether they

Table 5. Number of birds experimentally infected by subcutaneous inoculation with 1000 PFU of WNV that were positive for RNA at necropsy >6 wk postinfection

Bird	Scientific name	*n*	WNV positive	Organs positive
Passeriformes				
Brewer's blackbird	*Euphagus cyanocephalus*	8	2	Spleen, lung
Brownheaded cowbird	*Molothrus ater*	22	6	Spleen, lung, kidney, sera
European starling	*Sturnus vulgaris*	5	0	
House finch	*Carpodacus mexicanus*	23	12	Spleen, lung, kidney
House sparrow	*Passer domestica*	9	3	Spleen, lung, kidney, sera
White-crowned sparrow	*Zonotrichia leucophyrys*	5	3	Spleen
Columbiformes				
Common ground dove	*Columbina passerina*	6	2	Spleen, kidney
Mourning dove	*Zenaida macroura*	4	0	
Total		82	28	34%

were chronic infections in birds that died from other causes. Even other members of the family Corvidae such as western scrub-jays occasionally survive experimental infection, and antibody-positive individuals have been collected in Bakersfield, verifying natural survival of WNV infected birds (unpublished data).

Our experimental infection studies showed that 34% of infected Passeriformes and Columbiformes that survived acute infection contained WNV RNA >6 wk post infection. Species such as house finches and white-crowned sparrows that exhibited high peak viremias (>7 $\log_{10}$ PFU) and mortality rates (>70%) during acute infection (Reisen et al. 2005a) frequently exhibited chronic infections among survivors. Therefore, it was not clear whether the 33 positive birds testing positive during late winter died from acute infection with WNV, indicating recent transmission, or were infected during the previous transmission season, died from other causes, and retained WNV RNA. In agreement with the notion that they were infected during the previous summer, many of winter positives had marginally positive, but repeatable and confirmable TaqMan Ct scores >35, indicating a low level infection. Birds that die during acute infections typically have Ct scores <30. Bird die-offs because of other causes may have contributed to the detection of chronic WNV infections. For example, an outbreak of salmonellosis documented among pine siskins at winter bird feeders (http://www.dfg.ca.gov/news/news05/05011.htmL) resulted in 88 submissions for necropsy and testing, from which seven tested WNV positive. In this situation, as in the 26 other positive submissions, complete necropsies were not done to ascertain the cause of death.

All other surveillance indicators, including infection in diapausing and reproductively active mosquitoes, seroconversion by sentinel chickens, and reported cases of equine or human disease, remained negative at our study areas during both winters. Mosquito testing focused on bird feeding *Culex* vectors, however, mammal feeding *Culiseta* and *Aedes* also were tested with negative findings. Similar negative results were recorded throughout California during this period, with the exception of a positive pool of *Cx. p. quinquefasciatus* collected in Orange County and a single seroconversion in a sentinel chicken in San Bernardino during January 2005 (Arbovirus Bulletin #3, 25 February 05, California Department of Health Services). These positives may have represented the end of virus activity during the 2004 season, because the testing of 1,379 mosquitoes in 351 pools and 2,067 sentinel chicken sera from flocks bled biweekly throughout California from 1 January through 1 April 2005 yielded negative findings. Historically, pools of *Cx. tarsalis* from Coachella Valley have tested positive for WEEV and SLEV during January to March, only to be followed by a >2-mo interruption of transmission until summer (Reisen et al. 1992a).

Studies with nondiapausing eastern *Cx. p. pipiens* indicated that orally infected females were capable of maintaining WNV for up to 42 d at 10°C and that increasing temperature to 26°C activated virus and facilitated transmission (Dohm and Turell 2001). These data were supported by similar previous studies with western equine encephalomyelitis (family *Togaviridae*, genus *Alphavirus*, WEEV) and for St. Louis encephalitis virus (family *Flaviviridae*, genus *Flavivirus*, SLEV) in *Cx. tarsalis* and *Cx. p. quinquefasciatus* (Bellamy et al. 1967, 1968; Reisen et al. 1993b, 2002a). However, our recent temperature experiments with WNV indicated that the extrinsic incubation period of WNV was markedly elongated at 18°C (Reisen et al. 2006), making continued transmission strongly dependent upon elongated mosquito survival and periods of elevated winter temperature. Examination of temperatures in upland Los Angeles (Monrovia) and Coachella Valley indicated that winter temperatures were warm enough for continued winter transmission and that ample numbers of reproductively active and host-seeking females were present during the January–March period. However, infected mosquitoes were not found in Coachella Valley until 14 April 2004 (Lothrop 2005) and Los Angeles until 5 May 2004 (Wilson 2005).

In summary, continued WNV transmission was detected during winter by infections in dead American crows, but it seemed to be an infrequent event even in warm upland Los Angeles. Interruption of transmission by *Cx. tarsalis* in Coachella Valley was expected because of the photoperiod induced and maintained diapause that interrupted blood feeding even at warm latitudes (Reisen et al. 1995b). Another important enzootic vector, *Cx. stigmatosoma*, undergoes diapause in Oregon (Skultab and Eldridge 1985), but it has not been studied in southern California. *Cx. p. quinquefasciatus* does not undergo diapause (Reisen et al. 1986a) and gravid females were collected throughout winter in Los Angeles indicating continued gonotrophic activity. Failure to find infected females during this period perhaps relates to the dampening effects of cool temperature on transmission and to the importance of highly susceptible vector species such as *Cx. tarsalis* and *Cx. stigmatosoma* in enzootic amplification.

Vertical Transmission. Our field studies detected vertical transmission in *Cx. p. quinquefasciatus* during the summer transmission period in both Los Angeles and Kern counties. To our knowledge, these findings represent only the second report of WNV vertical transmission in nature and the first report documenting passage by WNV by *Cx. pipiens* complex females. Previously WNV was isolated from a pool of male *Cx. univittatus* collected in Kenya (Miller et al. 2000). However, WNV infection of diapausing *Cx. p. pipiens* females (Nasci et al. 2001, Bugbee and Forte 2004, Farajollahi et al. 2005) presumably occurred by the vertical transmission route. Although alternative infection mechanisms such as gonotrophic dissociation have been proposed to explain similar findings with SLEV (Bailey et al. 1978, Eldridge and Bailey 1979), subsequent physiological studies have refuted this infection mechanism (Mitchell 1981, Mitchell and Briegel 1989). If *Culex* destined for diapause indeed refuse to blood feed, then vertical infection is the only

mechanism whereby diapausing females may become infected.

Vertically infected males may be a dead end host for WNV. Although we were able to demonstrate venereal transmission of WNV after forced mating in two experiments, venereally infected females did not amplify virus after mating (titers in infected females remained low), and the single female that retained WNV for 3 d and oviposited did not pass this infection on to her progeny. We know that sperm was transferred to all females during artificial copulation, because all egg rafts hatched. Female engorgement before mating was shown to enhance venereal transmission by *Aedes* (Thompson 1983), perhaps indicating that bloodfeeding females in our study may have enhanced our chances of detecting venereal transmission. Previously, SLEV (Shroyer 1990) and dengue virus (Rosen 1987b) (both flaviviruses) were shown to be transmitted venereally by *Cx. p. quinquefasciatus* and *Ae. albopictus*, respectively, but the fate of these female infections was not resolved. In contrast, *Aedes triseriatus* (Say) females infected venereally with La Crosse virus (family *Bunyaviridae*, genus *Bunyavirus*) were shown to pass virus vertically to their progeny and horizontally to vertebrate hosts during blood feeding (Thompson and Beaty 1977, Thompson and Beaty 1978), thereby providing an additional amplification mechanism.

Chronic Infection. Tests on avian tissues taken at necropsy >6 wk after experimental infection showed that a high percentage of birds contained WNV RNA; sera from a single house sparrow also was positive. The form of WNV in these persistent infections was not clear, but infectious virus was recovered by Vero cell plaque assay from four of six RT-PCR-positive house finches after passage of organ tissue extracts through C6/36 *Ae. albopictus* cell culture. Although the frequency of chronic infection occurred at much lower rates, infectious WEEV and SLEV also has been recovered from necropsy material after similar blind passage (Reisen et al. 2001, 2003c). Potentially, stress associated with mating and territoriality, migration, or simply seasonal changes in hormone levels could trigger relapses in these chronically infected birds. Although not well documented for arboviruses, a change in photoperiod was found to stimulate recrudescence in *Borrelia*-infected birds of several species (Gylfe et al. 2000) and *Plasmodium*-infected house sparrows (Beaudoin et al. 1971). However, our previous attempts to stimulate relapses of WEEV and SLEV through experimental immunosuppression have failed (Reisen et al. 2003b, 2004a), and even birds negative for neutralizing antibody by plaque reduction neutralization test were found to be protected after challenge with conspecific virus (McLean et al. 1983, Reisen et al. 2003a). Therefore, although viral RNA and perhaps even infectious virus persists in bird organs, mechanisms that allow relapse and avoidance of the memory cell response have yet to be discerned.

Summary. Our research during two winters in California provided limited evidence to support three mechanisms of WNV persistence. Continued transmission was shown by the recovery of WNV RNA from dead American crows during winter but not by detection of infected mosquitoes or seroconversions in sentinel chickens. Vertical transmission was demonstrated in the laboratory but not in the field for *Cx. tarsalis* collected during summer and winter. In contrast, infected males and adults emerging from field-collected immatures were found in *Cx. p. quinquefasciatus*, indicating vertical transmission may be possible during mild winter conditions in southern California. WNV RNA was frequently detected at necropsy >6 wk postinfection in eight species of birds that were experimentally infected. These data perhaps explained the repeated detection of low level WNV RNA in dead bird species other than corvids that were tested during winter.

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