Migratory Birds and the Dispersal of Arboviruses in California

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Abstract. Each spring large numbers of neotropical migrants traversing the Pacific flyway pass through the Coachella Valley enroute to northern destinations, providing an opportunity to test the hypothesis that mosquito-borne encephalitis viruses are introduced annually into California by migratory birds. A total of 5,632 sera were collected from 43 species of migrants during spring (April–June), of which 34 (0.61%) comprised of 14 species tested positive by enzyme immunoassay; only 10 were confirmed by plaque reduction neutralization tests (PRNT). In addition, of 1,109 migrants comprised of 76 species that were reported dead by the public and necropsied, 126 (11%) were positive for West Nile virus (WNV) RNA; however, only three (0.7%) of 428 birds tested during the spring were positive. Limited experimental infection studies with WNV showed that Orange-crowned Warblers were highly susceptible and frequently died, whereas most Yellow Warblers survived. Our results indicated that birds entering California rarely exhibited a history of infection and that most birds probably became infected after entering California.

INTRODUCTION

The rapid dispersal and colonization of the New World by West Nile virus (WNV) has refocused attention on the role of migratory birds as vehicles for the rapid long distance movement of arboviruses.^{1–4} Proof of principal for long distance movement was shown recently in the Old World by the recovery of WNV from migrating storks⁵ and WNV antibody from other migrant species.^{6,7} Historically in the United States, large studies along the Mississippi and Atlantic flyways failed to document the introduction of encephalitis viruses by northbound migrants.^{8–11} In agreement, genetic studies have shown marked separation between North and South American strains of arboviruses, such as eastern equine encephalomyelitis (EEEV),¹² western equine encephalomyelitis (WEEV),^{13,14} and St. Louis encephalitis (SLEV)^{15,16} viruses, suggesting limited genetic exchange. Temporal studies in California have shown intermittent genotype change among WEEV and SLEV isolates,^{17–19} indicating possible introduction and replacement events. Neotropical migratory birds would seem to be the likely transport mechanism for such introductions.

The north shore of the Salton Sea in Coachella Valley is a unique area to study the introduction of arboviruses into western North America by northbound migrants. Typically, this is the first location in California and generally the western United States, where arboviral infections are discovered in mosquitoes each year,^{19–21} and the Imperial Valley just south of this location is where WNV was first discovered west of the continental divide.²² Large numbers of neotropical migrants transiting the Pacific flyway use marshes at the north shore as a stopover site after traversing the Salton Sea and adjacent desert facilitating capture. In addition, arboviruses are active enzootically during most summers at wetlands surrounding the Salton Sea, indicating amplification after local overwintering or introduction.

From 1996 through 2007, we sampled both migratory and resident birds for the presence of antibody or viral infection at the north shore of the Salton Sea each spring to test the

hypothesis that northbound migrants repeatedly introduce arboviruses into California. Similar data concurrently collected in Kern County at the southern end of the Central Valley provided an interesting comparison and a slightly different faunal composition. To assist in the interpretation of our field serology data, we experimentally infected Orange-crowned Warblers (*Vermivora celata*), Common Yellowthroats (*Geothlypis trichas*), Yellow Warblers (*Dendroica petechia*), and Yellow-rumped Warblers subspecies auduboni (*Dendroica coronata auduboni*) with either WNV or SLEV to measure viremia and mortality responses.

MATERIALS AND METHODS

Bird collections. Migratory and resident birds were captured while transiting sites on the northeastern (35.51, –115.92) and northwestern (33.46, –116.06) shores of the Salton Sea, Coachella Valley, Riverside County, CA, from 1996 through 2005. In addition, migratory transients were targeted during collections made during the springs of 2006 and 2007. Migrants and other birds also were collected during 1996 through 2008 in Kern County, primarily from wetlands at the Kern National Wildlife Refuge (35.73, –119.63) and the Kern River (35.32, –119.20). The ecology of these areas, descriptions of arbovirus activity, and bird sampling methods have been described previously.^{20,23} Briefly, birds were collected using multiple mist nets and grain-baited traps. Mist nets were 10 m long × 2.5 m tall and had 28-, 32-, or 60-mm mesh depending on the target species. Captured birds were banded with U.S. Geological Survey (USGS) bands, aged, sexed, and a 0.1 mL sample of blood collected from the jugular vein with 28-g needles. For antibody detection, blood samples were expelled into 0.9 mL of 0.9% saline solution, clarified through centrifugation, and stored at –70°C. During 2004, 2006, and 2007 blood samples from migratory species collected in Coachella Valley were expelled into 0.4 mL of virus diluent (phosphate buffered saline, 15% fetal bovine sera, antibiotics) and tested for infectious virus and antibody. Data from the California WNV Dead Bird Surveillance Program²⁴ on neotropical migrants that were found dead by the public, sent for necropsy, and tested positive for WNV RNA were included for comparison.

Experimental infection. Four warbler species commonly included in our field collections were experimentally infected

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with either WNV or SLEV. Birds were mist netted in either the Coachella Valley or Kern County, banded, and pre-bled to assure that they were serologically negative for WNV, SLEV, and WEEV, and transported to the Arbovirus Field Station. Birds were held in a mosquito-proofed and air conditioned facility, provided water *ad libitum*, and fed live meal worms, finely ground trout food (Aqua Max Carnivorous, Purina Mills, St. Louis, MO), and cut citrus. After an acclimation period of 7 to 14 d, birds were inoculated subcutaneously in the cervical region with $\approx 1,000$ plaque forming units (PFU) of the NY99 strain of WNV or the Kern217 strain of SLEV. Birds were bled daily for 6 to 7 d by jugular puncture (50–100 μ L of blood taken by 28-gauge syringe and expelled into 450 or 400 μ L of virus diluent) to monitor viremia response. Additional birds inoculated with virus diluent served as maintenance and handling controls.

Virological and serological testing. Wild bird sera were screened for antibodies to flaviviruses (WNV and SLEV) and WEEV using an enzyme immunoassay (EIA), with positives confirmed and identified by plaque reduction neutralization tests (PRNT).^{25,26} Those EIAs, with a positive/negative antigen well optical density ratio > 2.0 , were considered to be confirmed if the sera neutralized $> 80\%$ of > 75 PFU of the specific virus tested for when grown on Vero cells in 6-well plates at a serum dilution of 1:20. To separate previous infection of WNV from SLEV, PRNT end-point titers had to be $\geq 4\times$ the competing virus. To determine viremia, sera were tested by standard Vero cell plaque assay.²⁷ Dead birds reported by the public were necropsied at the California Animal Health and Food Safety Laboratory at the University of California, Davis, and kidney samples sent to the Center for Vectorborne Diseases laboratory for testing for WNV RNA by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)²⁸ using previously published primers.²⁹

Ethics. The collection and infection of wild birds was done under University of California Davis IACUC Protocols 12880 and 12876, respectively, CA Resident Scientific Collection permit 801049-02 by the State of California Department of Fish and Game, Federal Fish and Wildlife permit MB082812-0, and Master Station Banding Marking and Salvage permit 22673 by the USGS. Infection of birds and testing for WNV and SLEV was done under BSL3 conditions approved by University of California Davis Environmental Health and Safety Biological Use Authorization protocol 0554 and U.S. Department of Agriculture (USDA) permit no. 47901.

RESULTS

Field collections. A total of 5,600 sera were collected from 43 species of neotropical migrants during spring (April–June) in Coachella Valley or Kern County, of which 34 migrants (0.61%) comprising 14 species tested EIA positive for arbovirus antibody (Table 1). Of the 34 EIA positives, 10 were confirmed by PRNT; i.e., had an EIA with a mean positive over negative antigen well optical density ratio > 2.0 and a PRNT $\geq 1:20$. PRNT results that were $\geq 1:20$, but could not be confirmed as either SLEV or WNV, were presented as flavivirus positive. Bullock's Orioles (10 EIA positive) and Ash-throated Flycatchers (4 EIA positive) were the spring migrant species most frequently positive for arboviral antibodies. In addition, 1,727 sera from the same 14 species were collected during the summer and/or fall months,

of which 12 (0.69%) were EIA positive. This percentage positive was not statistically different ($P > 0.05$) than the percentage EIA positive for the spring migrants. Of these, 5 and 3 from 642 Common Yellowthroat, 0 and 3 from 42 Black-headed Grosbeak, and 0 and 1 from 182 Bullock's oriole sera were positive for WEEV or flavivirus antibodies, respectively; all other species listed in Table 1 tested negative. Only the 3 Black-headed Grosbeak sera were confirmed by PRNT to be SLEV antibody positive. In contrast, 2,987 (5.3%) sera from 56,174 birds collected over the entire study were positive for arboviral antibodies (Table 1), an order of magnitude greater than spring neotropical migrants. These non-neotropical migrant birds included winter, summer, and year-round residents, which were likely exposed locally to arboviruses.

Most of the common migrant species were collected during spring and fall (Figure 1A), but some Yellow-rumped Warblers and Common Yellowthroats remained as winter and year-round residents, respectively, in Coachella Valley. Residents could not be separated from migrant Common Yellowthroats except by time of capture, and therefore the resident portion of the population may have contributed to their slightly elevated seroprevalence in the Coachella Valley. The capture of migrants during spring was accompanied by the increase of WNV positive pools from mosquitoes collected along the Salton Sea (Figure 1B). The number of pools tested (mostly *Culex tarsalis* Coquillett) was related directly to the bimodal pattern of mosquito abundance at marshes along the Salton Sea. Virus amplification continued into June and the numbers of positive pools detected was highest during summer, decreasing in September as the migrants appeared again on their southbound flight.

Recapture patterns of banded birds provided some indication of their survival and rate of movement through our

FIGURE 1. Monthly changes in (A) numbers of birds collected per month during 1996–2005 and (B) pools of mosquitoes collected and testing positive for West Nile virus (WNV) (WNV+) from 2004 to 2007 from Coachella Valley. Bird species remaining year round were indicated by dashed lines.

TABLE 1

Neotropical migratory bird species collected in Coachella Valley (COAV) and Kern County (KERN) during spring (April–June) and tested for antibody against arboviruses*

Species	Site	Total	EIA results		PRNT results			
			WEEV +	FLAV +	WEEV +	SLEV +	WNV +	FLAV +
Lesser Nighthawk	COAV	33	1	0	0	•	•	•
<i>Chordeiles acutipennis</i>	KERN	0	0	0	•	•	•	•
Western Wood-Pewee	COAV	57	0	0	•	•	•	•
<i>Contopus virens</i>	KERN	3	0	0	•	•	•	•
Ash-throated Flycatcher	COAV	29	0	0	•	•	•	•
<i>Myiarchus cinerascens</i>	KERN	37	1	3	0	0	2	0
Western Kingbird	COAV	25	0	0	•	•	•	•
<i>Tyrannus verticalis</i>	KERN	242	0	2	•	0	0	0
Warbling Vireo	COAV	276	1	0	0	•	•	•
<i>Vireo gilvus</i>	KERN	16	0	0	•	•	•	•
Tree Swallow	COAV	0	0	0	•	•	•	•
<i>Tachycineta bicolor</i>	KERN	106	0	0	•	•	•	•
Cliff Swallow	COAV	0	0	0	•	•	•	•
<i>Petrochelidon fulva</i>	KERN	229	0	0	•	•	•	•
Swainson's Thrush	COAV	97	0	0	•	•	•	•
<i>Catharus ustulatus</i>	KERN	8	0	0	•	•	•	•
Hermit Thrush	COAV	13	0	0	•	•	•	•
<i>Catharus guttatus</i>	KERN	295	1	1	0	0	•	•
Orange-crowned Warbler	COAV	178	0	0	•	•	•	•
<i>Vermivora celata</i>	KERN	103	0	0	•	•	•	•
Nashville Warbler	COAV	47	1	0	0	•	•	•
<i>Vermivora ruficapilla</i>	KERN	14	0	0	•	•	•	•
Yellow Warbler	COAV	663	0	1	•	0	1	0
<i>Dendroica petechia</i>	KERN	60	0	0	•	•	•	•
Townsend's Warbler	COAV	18	0	0	•	•	•	•
<i>Dendroica townsendii</i>	KERN	3	0	0	•	•	•	•
MacGillivray's Warbler	COAV	139	0	2	•	0	1	0
<i>Oporornis tolmiei</i>	KERN	14	0	0	•	•	•	•
Common Yellowthroat	COAV	262	1	2	0	0	0	0
<i>Geothlypis trichas</i>	KERN	43	0	0	•	•	•	•
Wilson's Warbler	COAV	1,207	1	1	0	0	0	0
<i>Wilsonia pusilla</i>	KERN	118	0	0	•	•	•	•
Yellow-breasted Chat	COAV	33	0	0	•	•	•	•
<i>Icteria virens</i>	KERN	1	0	0	•	•	•	•
Summer Tanager	COAV	3	0	1	•	0	1	0
<i>Piranga rubra</i>	KERN	0	0	0	•	•	•	•
Western Tanager	COAV	74	1	0	0	•	•	•
<i>Piranga ludoviciana</i>	KERN	53	0	0	•	•	•	•
Black-headed Grosbeak	COAV	39	1	0	1	•	•	•
<i>Phenicus melanocephalus</i>	KERN	89	1	1	1	•	0	•
Blue Grosbeak	COAV	10	0	0	•	•	•	•
<i>Guiraca caerulea</i>	KERN	50	0	0	•	•	•	•
Lazuli Bunting	COAV	43	0	0	•	•	•	•
<i>Passerina amoena</i>	KERN	29	0	0	•	•	•	•
Yellow-headed Blackbird	COAV	83	0	0	•	•	•	•
<i>Xanthocephalus xanthocephalus</i>	KERN	8	0	0	•	•	•	•
Bullock's Oriole	COAV	44	1	1	0	0	•	•
<i>Icterus bullockii</i>	KERN	604	2	6	1	0	0	2
19 other species	COAV	69	0	0	•	•	•	•
	KERN	33	0	0	•	•	•	•
Total migrants	COAV	3,442	8	8	1	0	3	0
	KERN	2,158	5	13	2	0	2	2
Total Wild Birds [1996–2007]	COAV	26,708	201	663	91	119	248	88
[1997–2008]	KERN	29,466	386	1,737	140	1	1,407	196

*EIA = enzyme immunoassay; PRNT = plaque reduction neutralization test; WEEV = western equine encephalomyelitis; FLAV = flavivirus; WEEV = western equine encephalomyelitis; SLEV = St. Louis encephalomyelitis; WNV = West Nile virus. Not tested by PRNT.

study areas. Some abundant spring migrant species such as Wilson's and Yellow Warblers were never recaptured, and birds banded elsewhere also were not recovered in our collections. In addition, birds banded in Coachella Valley were never recaptured in Kern County or *vice versa*. In contrast, we frequently recaptured small-sized birds such as Orange-crowned Warblers (26 recaptures) and Common Yellowthroats (90 recaptures) that spent longer time periods within our study areas. The single seroconversion documented for

a migrant was a male after hatching year Common Yellowthroat that tested negative on September 19, 2003 and then positive for flavivirus the following week; titers were too low to separate WNV from SLEV. Summer residents such as Bullock's Orioles (175 recaptures) and Western Kingbirds (27 recaptures) frequently were recaptured near nesting sites in Kern County. Overall, banded birds were recaptured on 12,590 occasions (22.4% of total), but only 379 of these (3%) were bird species listed in Table 1. Most of these were recap-

tured during summer or during the slower autumnal return, and not on their vernal northern flight. In marked contrast other species were frequently recaptured, for example, 3,092 White-crowned Sparrows, a winter resident, were captured in Coachella Valley during the study period, of which 561 (18.1%) were recaptures.

In an attempt to detect acute arbovirus infections during the spring of 2006 and 2007, blood samples from the Coachella Valley were collected in virus diluent, frozen immediately on dry ice, and tested for viremia by Vero cell plaque assay. Overall, 1,222 migrant and a few resident birds were tested for virus with negative findings (Table 2). Similar negative results were found for 196 warblers tested for virus in May 2004. Sera also were tested for antibody, of which 6 sera from migrants were arbovirus positive by EIA (Table 3); one Yellow Warbler and one Magillivray's Warbler were confirmed by PRNT to be WNV positive in 2007. In addition, during 2007 three House Finches and 1 Gambel's Quail also were confirmed to be seropositive for WNV; all were after hatching year resident bird species and most likely were infected during the previous transmission season.

Dead bird testing. From 2003 through 2007, a total of 27,194 birds comprising 227 species were reported dead by the public in California and tested for WNV RNA, of which 33% were WNV positive. Most of the birds tested were resident corvid species. Of the 1,109 birds comprised of 76 migratory species, 11% were positive for WNV RNA (Table 4); however, only 3 (0.7%) of 428 birds tested during spring were positive (none from Coachella Valley), agreeing well with our serological results. The percentage of migrants positive for WNV RNA during spring (0.7%) was significantly less ($\chi^2 = 32.7$, $P < 0.001$) than other species found dead and tested during spring (493 pos [8.4%] of 5,886 tested). In addition, the percentage of migrants positive for WNV during spring (0.7%) was significantly less ($\chi^2 = 76.6$, $P < 0.001$) than the percentage of migrants found dead and testing positive during summer (18.1%). The Western Tanager represented this data trend well, with 1 of 31 positive during spring, but 22 of 78 positive during summer ($\chi^2 = 8.3$, $P < 0.004$). Collectively, these data indicated that migrants were becoming infected after they entered California.

Experimental infections. Four warbler species in three different genera within the family *Parulidae* were infected experimentally: Orange-crowned Warbler, Yellow Warbler, Common Yellowthroat, and Yellow-rumped Warblers subspecies *auduboni* (Figures 2 and 3). We had intended to use 6–8 birds per species per virus; however, we encountered difficulty adapting some species to captivity. We initially tried individual birds to learn holding and feeding methods and then tried to use these as “teacher” birds. Some species such as Orange-crowned Warbler adapted well, whereas others such as the Common Yellowthroat and Yellow-rumped Warbler did not. The single Common Yellowthroat used (Figure 2C) was our initial teacher bird, a second year male that survived more than 6 weeks in captivity; additional Common Yellowthroats and Yellow-rumped Warblers did not survive adaptation.

Orange-crowned Warblers were the most susceptible of the three species tested for WNV, producing the highest mean maximum viremia (mean = 8.45, range = 7.6–10.5 log₁₀ PFU/mL), longest duration viremia (max = 7 d), and most frequent mortality following infection (5 of 6 birds died by 7 d post infection) (Figure 2A). The single survivor had the lowest

TABLE 2
Resident and migratory birds collected in Coachella Valley during the spring of 2006 and 2007 and tested for viremia and antibody*

Common name	Scientific name	2006	2007	Total
Gambel's Quail	<i>Callipepla gambelii</i>		17	17
Mourning Dove	<i>Zenaida macroura</i>		1	1
Common Ground-Dove	<i>Columbina passerina</i>	13	7	20
Lesser Nighthawk	<i>Chordeiles acutipennis</i>	4		4
Western Wood-Pewee	<i>Contopus sordidulus</i>	4	14	18
Willow Flycatcher	<i>Empidonax traillii</i>	8		8
Hammond's Flycatcher	<i>Empidonax hammondi</i>	1		1
Dusky Flycatcher	<i>Empidonax oberholseri</i>		4	4
Pacific-slope Flycatcher	<i>Empidonax difficilis</i>	1	34	35
Black Phoebe	<i>Sayornis nigricans</i>	1		1
Ash-throated Flycatcher	<i>Myiarchus cinerascens</i>	2	10	12
Western Kingbird	<i>Tyrannus verticalis</i>	2		2
Cassin's Vireo	<i>Vireo cassinii</i>		2	2
Warbling Vireo	<i>Vireo gilvus</i>	12	69	81
Northern Rough-winged Swallow	<i>Stelgidopteryx serripennis</i>	2		2
Barn Swallow	<i>Hirundo rustica</i>		1	1
Verdin	<i>Auriparus flaviceps</i>	4	9	13
Cactus Wren	<i>Campylorhynchus brunneicapillus</i>		1	1
House Wren	<i>Troglodytes aedon</i>		2	2
Black-tailed Gnatcatcher	<i>Poliophtila melanura</i>	2		2
Swainson's Thrush	<i>Catharus ustulatus</i>	4	17	21
Northern Mockingbird	<i>Mimus polyglottos</i>	3	2	5
Orange-crowned Warbler	<i>Vermivora celata</i>	5	30	35
Nashville Warbler	<i>Vermivora ruficapilla</i>	1	1	2
Yellow Warbler	<i>Dendroica petechia</i>	73	100	173
Yellow-rumped Warbler	<i>Dendroica coronata</i>	1	2	3
Townsend's Warbler	<i>Dendroica townsendi</i>	2	8	10
Townsend's X Hermit Warbler Hybrid		1	1	2
Hermit Warbler	<i>Dendroica occidentalis</i>		1	1
Ovenbird	<i>Seiurus aurocapillus</i>		1	1
MacGillivray's Warbler	<i>Oporornis tolmiei</i>	37	37	74
Common Yellowthroat	<i>Geothlypis trichas</i>	6	10	16
Wilson's Warbler	<i>Wilsonia pusilla</i>	314	189	503
Yellow-breasted Chat	<i>Icteria virens</i>	1	7	8
Summer Tanager	<i>Piranga rubra</i>	2		2
Western Tanager	<i>Piranga ludoviciana</i>	11	21	32
Clay-colored Sparrow	<i>Spizella pallida</i>		1	1
Song Sparrow	<i>Melospiza melodia</i>	2	3	5
Lincoln's Sparrow	<i>Melospiza lincolni</i>	1	5	6
White-crowned Sparrow	<i>Zonotrichia leucophrys</i>	3		3
Golden-crowned Sparrow	<i>Zonotrichia atricapilla</i>		1	1
Black-headed Grosbeak	<i>Pheucticus melanocephalus</i>	1	9	10
Blue Grosbeak	<i>Guiraca caerulea</i>	1	2	3
Lazuli Bunting	<i>Passerina amoena</i>	7	3	10
Red-winged Blackbird	<i>Agelaius phoeniceus</i>		1	1
Great-tailed Grackle	<i>Quiscalus mexicanus</i>	1	1	2
Brown-headed Cowbird	<i>Molothrus ater</i>	3	7	10
Hooded Oriole	<i>Icterus cucullatus</i>	1		1
Bullock's Oriole	<i>Icterus bullockii</i>	2		2
House Finch	<i>Carpodacus mexicanus</i>		52	52
Total		539	683	1222

* Grain-baited trap was operated only during 2007.

viremia among the 6 birds tested. Two control birds survived the experimental period, which included daily blood sampling. In marked contrast, four Yellow Warblers and the single Common Yellowthroat produced significantly lower ($t = 2.79$, degrees-of-freedom [df] = 9, $P = 0.01$) mean maximum viremias (mean = 6.76, range = 5.6–7.5 log₁₀ PFU/mL), a shorter viremia period (3–4 d post infection), and a lower mortality rate (1 of 5 birds died) (Figure 2 B and C) than the

TABLE 3

Migratory birds collected in Coachella Valley during the spring of 2006 and 2007 that tested positive for antibody to WEEV or flaviviruses (Flav) by enzyme immunoassay (EIA) or plaque reduction neutralization assay (PRNT)

Species	Virus	EIA	PRNT
2006			
Summer Tanager	Flav	3.1	< 1:20
Western Tanager	WEEV	3.2	< 1:20
Wilson's Warbler	WEEV	2.0	< 1:20
Wilson's Warbler	Flav	2.3	< 1:20
2007			
MacGillivray's Warbler	WNV	7.5	> 1:80
Yellow Warbler	WNV	3.4	1:40

Orange-crowned Warblers. The single Yellow Warbler that died on Day 6 post infection was viremic until death, although the viremia was markedly lower during the two days just before death (Figure 2B). One non-infected Yellow Warbler control survived handling and blood collection.

In marked contrast to infection with WNV, 4 of 4 Orange-crowned Warblers infected with SLEV produced peak viremias on Days 1–2 post infection, cleared their viremia by Day 3, and survived (Figure 3A). All birds were subsequently bled weekly

TABLE 4

Spring–fall transients found dead by public and submitted to the Dead Bird Program for testing, 2003–2007*

Species	WNV – spring	WNV + spring	WNV – summer	WNV + summer	Grand total
Bank Swallow				1	1
Black-throated Gray Warbler				1	1
California Gull			8	1	9
Common Nighthawk			3	1	4
Flamingo†				1	1
Merlin				1	1
Peregrine Falcon				1	1
Townsend's Warbler			7	1	8
Tree Swallow			7	1	8
Virginia Rail			11	1	12
Warbling Vireo			5	1	6
Willow Flycatcher			1	1	2
Hooded Oriole			20	2	22
Lesser Nighthawk			5	2	7
Sora			12	2	14
Western Kingbird			27	2	29
Western Sandpiper			3	2	5
Common Yellowthroat			3	3	6
Hermit Thrush			24	3	27
House Wren			8	3	11
Osprey			1	3	4
Barn Swallow	19	1	25	4	49
Bullock's Oriole			13	4	17
Cliff Swallow	18	1	20	4	43
Orange-Crowned Warbler			13	4	17
Wilson's Warbler			17	4	21
Band-tailed Pigeon			15	5	20
Swainson's Hawk			12	5	17
Yellow Warbler			23	6	29
Rufous Hummingbird			9	7	16
Swainson's Thrush			55	7	62
Black-headed Grosbeak			106	17	123
Western Tanager	30	1	56	22	109
22 WNV neg. species summer			46		46
43 WNV neg. species spring	361				361
Grand total	428	3	555	123	1109

* Dead birds turned in to the California dead bird program 2003–2007 and tested for West Nile virus (WNV). Only birds that tested positive for WNV at least once were included in the table. The spring period is March 1–June 15 and summer period is June 16–September 30.

† Exact species unknown.

FIGURE 2. Experimental infections of (A) Orange-crowned Warblers, (B) Yellow Warblers, and (C) Common Yellowthroats with the NY99 strain of West Nile virus (WNV). Shown are daily viremia estimates in days post infection in $\log_{10}$ plaque forming units per mL for each bird dying (filled points) or surviving (open points). Assay sensitivity $2.0 \log_{10}$ plaque forming units (PFU)/mL.

for 6 weeks to follow antibody production. Antibody was readily detectable from Week 2–6 post infection, with mean EIA positive over negative antigen well ratio values ranging from 5.3 to 8.6. PRNT titers on Weeks 4 and 6 ranged from 1:20 to 1:40. In marked contrast, the single Yellow-rumped Warbler developed peak viremia between 5.3 and 7.4 $\log_{10}$ PFU/mL from Days 2–5 and then died (Figure 3B). Although our data were limited, they were interesting in that Orange-crowned Warblers, a warbler in the genus *Vermivora*, succumbed to WNV infection but survived SLEV, whereas warblers in the genus *Dendroica* survived WNV and one Yellow-rumped Warbler seemed to succumb from SLEV infection. Additional research is needed to improve these sample sizes and more fully understand the response of these warblers to flavivirus infection.

FIGURE 3. Experimental infections of (A) Orange-crowned Warblers and (B) Yellow-rumped Warblers with the KERN217 strain of SLEV. Shown are daily viremia estimates in days post infection in $\log_{10}$ plaque forming units per mL for each bird dying (filled points) or surviving (open points). Assay sensitivity $1.7 \log_{10}$ PFU/mL.

DISCUSSION

West Nile virus infections in *Cx. tarsalis* mosquitoes collected at marshes along the NE margin of the Salton Sea in Coachella Valley, Riverside County, repeatedly were detected as early as April (Figure 1B) and usually were the first detection of encephalitis virus in California.^{19–21} However, the source of these early season infections has yet to be determined. The recovery of multiple WNV-positive pools coincidental with the arrival of the first migrants made them suspect as the source of infection; however, there was no further increase in positive pools during May when most of the migrants passed through the Coachella Valley. In addition, over an 11-year period, few avian migrants entering California from southern overwintering locations were antibody positive, indicating a limited history of previous viral exposure. These data were consistent with the “disappearance” of WNV and other temperate arboviruses in the neotropics.^{30,31} Portions of some migrant populations such as the Common Yellowthroats remained in the Coachella Valley during summer (Figure 1A) and therefore may have been infected locally at that time as indicated by the single seroconversion during September 2003. Local infection also seemed evident from the dead bird data shown in Table 4.

Several migrants had low level EIA antibody that could not be confirmed by PRNT. These may be old infections, because we previously reported SLEV antibody decay in House Finches and Mourning Doves following experimental infections, with antibody detected by EIA in birds testing negative by PRNT the spring following the year of infection.^{32,33} In contrast, WNV produces long-lasting and elevated PRNT

titers in House Sparrows and House Finches, perhaps stimulated by the intermittent release of viral antigen from long-lasting chronic infections.^{34–37} During 2004, 2006, and 2007, we also tested these same migrant species for viremia to evaluate the notion that they may become infected during the northern migration and arrive viremic, but before becoming antibody positive. However, all these birds were negative by Vero cell plaque assay. This was unexpected because WNV was detected in *Cx. tarsalis* during early spring of both 2006 and 2007.³⁸

Interestingly, some migrant species found dead by the public had kidney tissue collected at necropsy positive for WNV RNA by qRT-PCR. Dead birds reported as positive either had Ct scores < 30 for a primer set specific for the envelope region of the viral genome or if the Ct score was between 30 and 40, these infections were confirmed by qRT-PCR using a second primer set specific for the non-structural-NS3 gene of the WNV genome. Therefore, WNV-positive dead birds could have either succumbed during acute infection acquired locally or survived and maintained viral RNA in their kidney tissues. Previously, we reported evidence for persistent viral RNA in birds necropsied 6–8 weeks after experimental infection, and similar to hamsters,³⁹ some of these tissues yielded infectious virus after C6/36 cell passage.^{36,40} One of the four Orange-crowned Warblers infected with SLEV had brain tissue positive at necropsy 6 weeks after experimental infection.

The percentage of all dead birds reported by the public that were positive for WNV at necropsy was an order of magnitude greater than the proportion of live birds that tested positive for antibody against WNV. Although all three species of warbler we experimentally infected were competent hosts for WNV, they differed markedly in the amplitude of their acute viremia and mortality rates. Orange-crowned Warblers readily succumbed to WNV, but not SLEV, experimental infection, and in agreement 11% of Orange-crowned Warblers found dead were WNV positive, whereas no live birds tested positive for WNV antibody. The single Common Yellowthroat experimentally infected with WNV survived, and in agreement several birds were found with flavivirus or WNV antibody in both Coachella and Kern County; none were found dead by the public and submitted for testing.

The general lack of evidence for previous or current arboviral infection in migratory birds entering California from southern overwintering sites indicated that migrants reported positive by the dead bird program probably were infected locally rather than on their overwintering grounds. The passage of these migrants through Coachella Valley after virus infection was detected in *Cx. tarsalis* may indicate that these birds acquired their infection locally and then dispersed virus northward from southern California into the Central Valley. In agreement, during the summer of 2004, WNV rapidly moved > 800 km northward from the Coachella Valley and Los Angeles basin into every county in California.⁴¹

Our current research^{36,42} has yet to resolve the primary mechanism(s) of WNV overwintering in California or possibly temperate North America, leaving us with the same suite of possibilities presented almost 50 years ago.⁴³ Although a variety of these mechanisms have been explored since the arrival of WNV, all data were based on laboratory experiments or relatively rare field events that have been difficult to detect and repeat in nature. Spatial and temporal variation in the intensity of summer enzootic transmission levels undoubtedly has altered the magnitude of the overwintering virus

population, thereby adding to the complexity of resolving the mechanism(s) of virus overwintering in nature.

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