Detection of Persistent West Nile Virus RNA in Experimentally and Naturally Infected Avian Hosts

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Abstract. To determine whether West Nile virus (WNV) persistent infection in avian hosts may potentially serve as an overwintering mechanism, House Sparrows and House Finches, experimentally and naturally infected with several strains of WNV, and two naturally infected Western Scrub-Jays were held in mosquito-proof outdoor aviaries from 2007–March 2008. Overall, 94% (n = 36) of House Sparrows, 100% (n = 14) of House Finches and 2 Western Scrub-Jays remained WNV antibody positive. When combined by species, 37% of the House Sparrows, 50% of the House Finches, and 2 Western Scrub-Jays were WNV RNA positive at necropsy, up to 36 weeks post-infection. Infectious WNV was not detected. Our study supports the hypothesis that some avian hosts support the long-term persistence of WNV RNA, but it remains unresolved whether these infections relapse to restart an avian-arthropod transmission cycle and thereby serve as an overwintering mechanism for WNV.

INTRODUCTION

West Nile virus (*Flaviviridae: Flavivirus*, WNV) is an enveloped, single-stranded, positive-sense RNA virus.¹ Like other members of the Japanese encephalitis serocomplex, it is maintained in an enzootic cycle among free-ranging birds, mostly within the order Passeriformes, and ornithophilic *Culex* mosquitoes. Humans and equines are infected tangentially to this primary cycle.² West Nile virus was first detected in the New World in 1999,³ and within five years spread across the United States, north into Canada and as far south as Argentina.⁴ In the wake of the North American invasion, WNV transmission has reappeared each summer; in 2010, for example, the Centers for Disease Control and Prevention reported WNV activity in every one of the continental United States (<http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm>). However, the mechanisms that enable WNV to overwinter when mosquito vectors are inactive and transmission subsides are not well understood, but may include persistent infections in mosquito vectors and/or avian hosts. The present investigation focuses on persistent WNV infection in avian hosts.

Herein, persistent WNV infection is defined as the detection of infectious virus or viral RNA in host tissues, after the acute viremia has subsided. In most birds, acute infection is accompanied by a viremia lasting 2–7 days, depending on the hosts' susceptibility to WNV. These infections generally either end in subsidence of the viremia, production of antibodies, and survival, or in an uncontrolled viremia and death.^{5,6} Raptors, however, such as Great Horned Owls (*Bubo virginianus*) and Red-tailed Hawks (*Buteo jamaicensis*), may clear the initial viremia, but suffer long-term sequelae impairing their ability to survive in the wild.⁷ Evidence of WNV persistence has been reported in several mammalian models and in convalescing human patients. Golden hamsters (*Mesocricetus auratus*) experimentally infected with WNV shed virus in their urine for up to eight months post-infection

(pi), developing chronic renal infections.⁸ Similarly in immunocompetent B-6 mice (*Mus musculus*), infectious WNV persisted in tissues for up to four months pi, and WNV RNA was detected for up to six months pi.⁹ Viral antigen was detectable for up to 5.5 months in rhesus monkeys (*Macaca mulatta*) experimentally infected with WNV.¹⁰ Similar to the finding in hamsters, WNV RNA, but not infectious virus, was reported in 20% (n = 25) of urine samples, collected from human patients convalescing from WNV infection, 1.6–6.7 years pi.¹¹

Persistent WNV infections also have been reported in birds. Early work by Semenov and others¹² described persistent WNV infections in Blue-Gray Pigeons (*Columba cf. livia*), with virus isolated from blood 16, 93, and 100 days pi (dpi), and viral antigen detected in liver imprints up to 184 dpi. Antibody titers fluctuated from non-detectable to detectable, but were low-grade (1:40 by hemagglutination inhibition reaction). Interestingly, on two occasions, the reappearance of increased antibody titers was associated with a recrudescence of viremia.¹² Reisen and others¹³ reported that 6 of 8 bird species held 6–8 weeks post-WNV experimental infection maintained WNV RNA in tissues, with the spleen and kidney most commonly positive. A subset of House Finches (*Carpodacus mexicanus*) found positive for WNV RNA were also positive for infectious WNV by Vero cell plaque assay after passage in C6/36 cells.¹³ Nemeth and others¹⁴ reported similar results for House Sparrows (*Passer domesticus*), with WNV RNA detected in spleen and/or kidney tissues for up to 65 days and infectious virus detected in the spleen up to 43 dpi. In contrast, Gray Catbirds (*Dumetella carolinensis*) failed to show persistent infection with WNV.¹⁵ Studies with St. Louis encephalitis virus (SLEV), a virus endemic to North America that is also in the Japanese encephalitis serocomplex, have yielded mixed results. This virus was isolated from the gizzard of an experimentally infected Brown-headed Cowbird (*Molothrus ater*) 38 dpi,¹⁶ but was not isolated from 369 birds comprising 9 species necropsied more than 7 dpi.¹⁷ In contrast, SLEV RNA was recovered from 1 of 14 experimentally infected House Finches by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) one year pi.¹⁸

Although persistent infections have been reported in avian hosts, thus far, aside from the findings in pigeons,¹² the

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duration of WNV persistence in mammals has far exceeded persistence in birds. However, persistent infection in avian hosts may be an important factor enabling WNV to overwinter and initiate transmission in the spring, when few *Culex* mosquitoes feed on mammals.^{19,20} To determine whether WNV infections can persist overwinter in California, House Sparrows and House Finches experimentally infected with WNV were held under ambient conditions (temperature range = 5°C–36°C) within mosquito-proof outdoor aviaries in Kern County, CA (35°24'13.45"N, 119°08'50.03"W) during September–March (overwinter). Limited numbers of naturally infected House Sparrows (n = 9), Western Scrub-Jays (n = 2) and 1 House Finch were collected in Kern County and held concurrently. The study was terminated in spring when birds were necropsied and tested for WNV-neutralizing antibodies and the presence of persistent WNV RNA and infectious virus.

MATERIALS AND METHODS

Birds. Birds were collected during the spring of 2007 by mist net or grain-baited trap²¹ in Kern County. All birds were first screened for antibodies against western equine encephalomyelitis virus (WEEV), SLEV, and WNV by using an enzyme immunosorbent assay²²; birds with positive results by this assay were confirmed by using a plaque-reduction neutralization test (PRNT).²³ All birds were negative for WEEV and SLEV. Birds with positive results for WNV by PRNT had survived natural infection and were retained for comparison. Antibody-negative birds were experimentally infected during the summer (June–September) of 2007, held over the winter of 2007–2008, and then necropsied in the spring (March) of 2008. Two weeks pi and after clearance of acute viremia, birds were moved into mosquito-proof, outdoor aviaries. Each species was housed separately in roofed enclosures that were furnished with perches, nest boxes, water baths, and a mixture of commercially available wild bird seed.

Experimental design. Birds necropsied in spring were survivors from a series of experimental infections with several WNV genotypes (Table 1). A subset of House Sparrows were infected with either the mouse neuroinvasive (BIRD 1461, TM171-03-pp5 and NY99) or non-neuroinvasive

(TM171-03-pp1 and BIRD1153) isolates of WNV; detailed descriptions of these genotypes and associated avian infection response phenotypes were reported by Brault and others.²⁴ The remaining House Sparrows were infected with a NY99 WNV infectious clone²⁵ containing one of five point mutations. Because sample sizes of birds available for analysis were limited, these viruses were evaluated as a group. House Finches were experimentally infected with either TM171-03-pp5 or NY99.

Birds were infected by subcutaneous needle-inoculation into the cervical area with 0.1 mL of virus diluent (phosphate-buffered saline, 15% fetal bovine serum, and antibiotics) containing approximately 1,000 plaque-forming units (pfu) of WNV, a biologically relevant dose reportedly expectorated by *Culex* mosquitoes.^{5,26} Blood was collected from infected birds on 1–7 dpi to assess viremia: 100 µL of blood was collected with 28-gauge needles by jugular venipuncture, diluted in 400 µL of virus diluent, and frozen at –80°C.

Birds that were WNV antibody positive at the time of collection were held in captivity for at least 4 months, whereas birds experimentally infected were retained for 6–8 months. Birds were euthanized by exsanguination (approved under Institutional Animal Care and Use Committee protocol 12876) and serum was used for antibody assays. Samples of spleen and kidney, and a full panel of tissues, were collected and fixed in 10% neutral-buffered formalin for histopathologic and immunohistochemical (IHC) analyses using reported methods.²⁷ The spleen was divided into three approximately equal portions; two samples were immediately frozen at –80°C and reserved for RNA extraction and virus isolation, and the remaining sample was formalin-fixed for histopathologic analysis and IHC. Two kidney samples with a volume of approximately 5 mm³ were frozen at –80°C and reserved for RNA extraction and virus isolation. To prevent cross-contamination, instruments were flame-sterilized and rinsed in 70% isopropyl alcohol between tissue samples.

Serologic analysis. Blood samples collected at necropsy were tested for WNV-specific neutralizing antibodies by a PRNT.²³ In brief, serum samples were heat inactivated at 56°C for 30 minutes and diluted in a two-fold series starting at 1:2.5. Diluted serum samples were mixed 1:1 with virus diluent containing approximately 100 pfu of WNV (NY99 strain

TABLE 1
Summary results for birds grouped by infecting WNV genotype*

Species†	WNV genotype	No.	Mean peak viremia‡	Geometric mean antibody titer§	PRNT ₉₀ antibody titer range	No. (%) WNV RNA positive¶
House Finch	NY99	5	7.1	1:557	1:320–1:1,280	3 (60)
	TM 171-03 ppl	10	3.9	1:43	1:5–1:160	5 (50)
	Naturally infected	1	NT	1:1280	1:1,280	0
	Overall	16	5	1:118	1:5–1:1,280	8 (50)
House Sparrow	NY99	2	6.2	1:160	1:80–1:320	1 (50)
	TM 171-03 ppl	9	2.9	1:174	< 1:5–1:1,280	3 (33)
	TM 171-03 pp5	3	5.0	1:254	1:80–1:640	1 (33)
	BIRD 1153	2	6.2	1:320	1:160–1:640	0
	BIRD 1461	4	5.8	1:190	1:40–1:640	0
	NY99 point mutant	6	3.6	1:202	1:40–1:640	4 (67)
	Naturally infected	9	NT	1:101	< 1:5–1:320	4 (44)
	Overall	35	4.3	1:169	< 1:5–1:1,280	13 (37)
Western Scrub-Jay	Naturally infected	2	NT	1:113	1:40–1:320	2 (100)

*WNV = West Nile virus.

†House Finch (*Carpodacus mexicanus*); House Sparrow (*Passer domesticus*); Western Scrub-Jay (*Aphelocoma californica*).

‡Log₁₀ plaque-forming units/mL of serum. Viremia titers of naturally infected birds were unavailable and not tested (NT).

§Tested by plaque reduction neutralization test (PRNT). Shown is the highest serum dilution at which 90% of > 75 plaque-forming units of WNV were neutralized. The lowest serum dilution tested was 1:5.

¶Spleen and kidney tissue were screened for WNV RNA by using quantitative reverse transcription–polymerase chain reaction and primers and probe specific for the envelope region of the viral genome (WN1).

35211 AAF 9/23/99, which was isolated from a moribund Flamingo in 1999). A double overlay system was used; the first overlay (nutrient medium, 1% agarose, and 3% sodium bicarbonate) was applied after the serum/virus mixtures were incubated for one hour on a Vero cell monolayer. The second overlay (nutrient media, 1% agarose, 3% neutral red, and 3% sodium bicarbonate) was applied 48 hours after the first overlay; the serum/virus mixture was incubated at 37°C for 72 hours. End point titers were determined as the highest dilution at which 90% of > 75 PFU were neutralized (PRNT₉₀); the highest serum concentration tested was 1:5.

Detection of WNV RNA. RNA was extracted by using an ABI PRISM™ 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA) and/or by using guanidinium thiocyanate-phenol-chloroform extraction using TRIzol® LS reagent (Invitrogen, Carlsbad, CA). To maximize RNA detection, the entire first tissue sample was used for RNA extraction. Extracted RNA were first screened for the presence of WNV by using a qRT-PCR and the 7900 TaqMan® platform (Applied Biosystems) according to the manufacturer's protocols and using primers and a probe specific for the envelope region of the viral genome (WN1)²⁸: (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-TAMRA. Samples with a cycle threshold (C_t) score < 40 were considered positive, and confirmation was attempted by using primers and probe specific for the non-structural protein 1 region of the viral genome (WN2)²⁹: (forward) 5'-GGC AGT TCT GGG TGA AGT CAA-3', (reverse) 5'-CTC CGA TTG TGA TTG CCT CGT-3', and (probe) 6FAM-TGT ACG TGG CCT GAG ACG CAT

ACC TTG T-TAMRA. Samples with a WN2 Ct value < 40 were considered positive. When possible, samples that were positive for WNV RNA using WN1 also were confirmed by a second RNA extraction and qRT-PCR using WN1.

Virus isolation. When available, avian tissues positive for WNV RNA were assayed for infectious virus (Table 2). Frozen tissues were thawed on ice and homogenized by using a hand-held tissue homogenizer (Omni International, Marietta, GA) in 1.5 mL of Dulbecco's modified Eagle medium (Invitrogen/Gibco, Grand Island, NY) containing 10% fetal bovine serum and 5% penicillin and streptomycin. Tissue homogenate (1.0 mL) was added to a 75-cm² tissue culture flask containing 90% confluent C6/36 *Aedes albopictus* cells from which all but 2.0 mL of the tissue culture medium had been removed. After a one-hour absorption period, the medium was replaced and the cells were incubated at 28°C for 7 days. Cells were collected with the tissue culture medium and centrifuged at 1,840 × g for 10 minutes at 4°C. The supernatants were tested for infectious virus by using a standard Vero cell (cell line derived from kidney epithelium of an African green monkey) plaque assay.³⁰ RNA also was extracted from the tissue homogenate and tested by using qRT-PCR and the WN1 primers and probe as described above.

Statistical analysis. Student's *t*-test was used to test for significant differences in neutralizing antibody responses between birds that were positive or negative for WNV RNA. For this analysis, serum samples collected at necropsy were tested by PRNT₉₀ as described above. Birds that had an antibody titer < 1:5 were considered to be 1:2.5; reciprocal PRNT₉₀ antibody titers were log₁₀ transformed before analysis. Fisher's exact test³¹ was used to test for significant differences in the

TABLE 2
Detailed assay results for birds positive for WNV RNA*

Bird no.	Species†	Weeks PI‡	WNV genotype	Mean peak viremia§	PRNT ₉₀ antibody titer¶	Organs positive for WNV RNA (C _t)#
434	House Finch	28	NY99	7.5 (3)	1:320	Spleen (33.6, UD)
435	House Finch	28	NY99	7.5 (3)	1:1280	Spleen (33.7, UD), kidney (28.8, 29.5)
436	House Finch	28	NY99	7.2 (3)	1:320	Spleen (31.6, UD)
408	House Finch	28	TM 171-03 pp1	4.3 (3)	1:80	Kidney (33.2, 35.2)
409	House Finch	28	TM 171-03 pp1	4.9 (3)	1:40	Spleen (33.3, UD)
414	House Finch	28	TM 171-03 pp1	4.0 (3)	1:80	Kidney (27.2, UD)
417	House Finch	28	TM 171-03 pp1	54 (4)	1:10	Spleen (35.2, 39.1)
423	House Finch	28	TM 171-03 pp1	1.7	1:5	Spleen (35.7, UD)
30	House Sparrow	> 18	Naturally infected	NT	1:320	Spleen (36.9, UD)
176	House Sparrow	> 18	Naturally infected	NT	NS	Spleen (34.3), kidney (29.9)
193	House Sparrow	> 18	Naturally infected	NT	< 1:5	Spleen (33.1)
308	House Sparrow	> 18	Naturally infected	NT	1:320	Spleen (32.2)
171	House Sparrow	28	NY99	6.4 (2)	1:320	Spleen (35.1)
256	House Sparrow	31	NY99IC point mutant	4.5 (4)	1:640	Spleen (31.0)
277	House Sparrow	31	NY99IC point mutant	4.7 (5)	1:320	Spleen (34.2), kidney (30.8)
300	House Sparrow	31	NY99IC point mutant	5.3 (2)	1:40	Spleen (32.6)
302	House Sparrow	31	NY99IC point mutant	2.7 (3)	1:320	Kidney (32.7)
207	House Sparrow	36	TM 171-03 pp1	1.7	1:1280	Spleen (33.7, UD)
163	House Sparrow	28	TM 171-03 pp1	5.1 (3)	1:640	Kidney (30.9)
168	House Sparrow	28	TM 171-03 pp1	2.4 (3)	1:320	Kidney (31.9)
182	House Sparrow	28	TM 171-03 pp1	1.7	< 1:5	Kidney (34.3)
209	House Sparrow	36	TM 171-03 pp5	5.0 (2)	1:320	Spleen (37.3, 38.3)
6845	Western Scrub-Jay	> 18	Naturally-infected	NT	1:320	Spleen (34.1, UD)
6852	Western Scrub-Jay	> 18	Naturally infected	NT	1:40	Spleen (33.7, UD), kidney (35.7, UD)

* WNV = West Nile virus.

† House Finch (*Carduelis mexicanus*); House Sparrow (*Passer domesticus*); Western Scrub-Jay (*Aphelocoma californica*).

‡ PI = post-infection. Time PI for naturally infected birds was unknown but was > 18 weeks post-collection.

§ Peak viremia was measured in log₁₀ plaque-forming units (pfu)/mL of serum by Vero cell plaque assay; day post-infection for which peak viremia was measured is shown in parentheses; NT = not tested; 1.7 log₁₀ pfu/mL was the detection limit for this assay.

¶ PRNT₉₀ = plaque reduction neutralization titer. Shown is the highest serum dilution at which 90% of > 75 plaque-forming units of WNV were neutralized. The highest serum dilution tested was 1:5.

Tissues were tested for WNV RNA by using reverse transcription-polymerase chain reaction and primers and a probe specific for the envelope region of the viral genome (WN1). Taqman cycle threshold (C_t) score is shown in parentheses. When two C_t scores are present, the second result was extracted from tissue homogenate used for viral isolation; UD = undetermined, i.e., C_t > 40.

proportion of persistently infected birds within species, comparing groups of birds by infecting genotype and mean peak viremia, and overall between House Sparrows and House Finches. Some experimentally infected birds failed to develop detectable viremias, and groups also were compared with these non-viremic birds omitted.

Ethics. The collection, housing, transport, infection, and euthanasia of birds were conducted under approved University of California, Davis, Institutional Animal Care and Use Committee protocols 12876 and 12880. Birds were collected by using grain-baited traps and mist nets under U.S. Geological Survey Master Station Banding Permit 22763 and State of California Scientific Collecting Permits, and taken for experimentation under Federal Permit MB082812. BSL3 laboratory facilities were approved under BUA 0554 by the University of California, Davis, Environmental Health and Safety Committee and USDA Permit 47901.

RESULTS

Serologic analysis. At termination, 100% ($n = 15$) of the House Finches and 97% ($n = 29$) of the House Sparrows had PRNT₉₀ antibody titers $\geq 1:5$ (Table 1). All of the naturally infected birds (1 House Finch, 2 Western Scrub-Jays, and 7 House Sparrows) maintained PRNT₉₀ endpoint titers $\geq 1:5$ (Table 1).

Persistence of WNV RNA. A summary of the test results for WNV RNA-positive birds is shown by leg band number in Table 2. Initially, RNA was extracted from the spleens and kidneys of 21 of 56 birds by using the ABI PRISM™ 6100 Nucleic Acid PrepStation; none were positive by qRT-PCR using WN1. Because these results were unexpected and different from those of our previous and on-going studies, RNA was re-extracted by using TRIzol reagent from the second tissue sample originally collected for virus isolation; in this instance, WNV RNA was detected in 9 of 21 birds. Therefore, all subsequent samples were extracted by using TRIzol reagent. When the experimentally and naturally infected birds were combined by species, 37% of the House Sparrows, 50% of the House Finches and 100% of Western Scrub-Jays had spleen and/or kidney tissues positive for WNV RNA. These birds were tested by using qRT-PCR with the WN1 primers and probe. When compared by using a series of Fischer exact tests, there were no significant differences ($P > 0.05$) in the proportion of RNA-positive birds among different WNV strains within House Finches and House

Sparrows or between these two species. After two freeze-thaw cycles, only one sample collected from the kidney of a House Finch (bird # 435) was WNV RNA positive when tested with the WN2 primers and probe.

Cycle threshold scores for birds positive for WNV RNA ranged between 29.9 and 38.3 (Table 2), corresponding to approximately 100–1 pfu of WNV/mL, respectively. When available, virus isolation was attempted from samples of WNV RNA positive tissues; overall, 16 tissues from 14 birds were tested and showed negative results. In an attempt to further confirm initial results after TRIzol extraction, RNA was extracted from these 16 tissue homogenates used for virus isolation and screened for WNV RNA by qRT-PCR (WN1), of which 19% were again WNV RNA positive when WN1 was used (Table 2). These tissue homogenates had C_t scores that were consistently higher than C_t scores derived from the first tissue sample, possibly because of extraction of RNA from smaller quantities of tissue and the additional freeze-thaw cycle.

No significant difference ($P > 0.05$) was detected in the geometric mean PRNT₉₀ titer of birds that were WNV RNA positive (geometric mean = 171) and those that were WNV RNA negative (geometric mean = 156). In addition, no significant difference ($P > 0.05$) was detected in the proportion of WNV RNA-positive birds between species and among groups of House Sparrows naturally infected or with different genotypes of WNV (whether or not non-viremic birds were included in the analysis) (Table 1). There was also no significant difference ($P > 0.05$) between the two groups of experimentally infected House Finches (the naturally infected group was not compared because it only contained one bird). To test for the effect of virulence on persistence, birds were grouped by species and mean peak viremia (Table 3), but again no significant differences ($P > 0.05$) were detected in the proportion of RNA-positive birds. However, relatively small sample size within groups and variation among replication phenotypes may have contributed to this lack of significance.

Histopathologic and IHC analyses. Initially, two birds were chosen for histopathologic and IHC analyses: a House Sparrow (bird #176) and a House Finch (bird #435) that had spleen and kidney tissues positive for WNV RNA. West Nile virus-specific IHC staining was not seen in either bird; therefore, no additional birds were tested. However, splenic lymphofollicular hyperplasia and myelitis in the bone marrow (multifocal, lymphocytic) were noted in the House Finch. The

TABLE 3
Summary results for birds grouped by mean peak viremia for WNV*

Species†	Peak viremia titer range	No.	Mean peak viremia‡	Geometric mean antibody titer§	PRNT ₉₀ titer range	No. (%) WNV RNA positive¶
House Finch	0–3.0	3	2.2	1:25	1:320–1:1,280	1 (33)
	3.1–5.0	6	4.5	1:71	1:5–1:160	3 (50)
	5.1–8.0	6	6.8	1:285	1:10–1:1,280	4 (67)
	Naturally infected	1	NT	1:1,280	1,280	0
House Sparrow	0–3.0	12	2.2	1:226	1:80–1:320	5 (42)
	3.1–5.0	6	4.4	1:160	< 1:5–1:1,280	3 (50)
	5.1–8.0	11	5.8	1:219	1:80–1:640	3 (27)
	Naturally infected	9	NT	1:101	1:160–1:640	4 (44)
Western Scrub-Jay	Naturally infected	2	NT	1:113	1:40–1:320	2 (100)

*WNV = West Nile virus.

†House Finch (*Carpodacus mexicanus*); House Sparrow (*Passer domesticus*); Western Scrub-Jay (*Aphelocoma californica*).

‡Mean peak viremia represented in log₁₀ plaque-forming units/mL of serum. Viremia titers of naturally infected birds were unavailable and not tested (NT).

§Tested by plaque reduction neutralization test (PRNT). Shown is the highest serum dilution at which 90% of > 75 plaque-forming units of WNV were neutralized. The lowest serum dilution tested was 1:5.

¶Spleen and kidney tissue were screened for WNV RNA by using quantitative reverse transcription-polymerase chain reaction and primers and a probe specific for the envelope region of the viral genome (WN1).

House Sparrow also showed inflammation in several different tissues: enteritis (moderate, diffuse, lymphoplasmatic), interstitial nephritis (multifocal, lymphocytic), myelitis (mild, multifocal lymphocytic), epicarditis (multifocal, lymphonodular), coelomitis (multifocal, lymphonodular), and bronchitis (multifocal, lymphonodular). At this time, it is unknown whether persistent WNV was the cause of the inflammatory changes, and further work will be needed to determine whether specific lesions are associated with WNV persistence.

DISCUSSION

West Nile virus RNA was detected up to 36 weeks pi in the spleens and kidneys of House Sparrows and House Finches that were either experimentally or naturally infected with WNV. These data confirmed the long-term persistence of WNV in an avian host reported by Semenov and others.¹² Recent studies of persistent WNV infections in avian species have detected virus or viral RNA at six weeks¹³ and up to 65 days¹⁴ pi. We report WNV RNA in tissues up to 36 weeks pi. This long-term persistence of WNV RNA was comparable to previous experimental results in mice,⁹ in which WNV RNA was detected up to six months pi, but in which infectious virus was not isolated.

West Nile virus-specific neutralizing antibodies were detected in the serum of experimentally and naturally infected birds at necropsy. The humoral immune response to WNV is long-lasting and protective in House Sparrows.³² However, it is unknown whether long-term shedding of WNV is associated with persistence of antibody titer. An increase in neutralizing antibody titers in conjunction with recrudescence viremia was detected in persistently infected pigeons; however, high levels of neutralizing antibody were not associated with detection of WNV RNA in the spleen or kidney.¹² Similar fluctuations in antibody titer were observed in Rock Pigeons (*Columba livia*) naturally infected with WEEV or SLEV,³³ but these birds were not tested for recrudescence viremia. In contrast, House Finches experimentally infected with SLEV progressively lost detectable PRNT antibody titer, and, in agreement, only 1 (7.7%) of 13 birds were positive for SLEV RNA at necropsy 6 months pi.¹⁸

Attempts at isolating infectious virus were not successful. Therefore, it was not determined whether the WNV RNA detected should be attributed to persistent infectious virus or residual RNA from inactivated virus from the initial infection. The method used to isolate virus after blind passage in C6/36 cells was successful in detecting infectious virus at six weeks pi.¹³ However, this method may not have been adequately sensitive at ≥ 6 months pi. All samples were frozen at -80°C until processing (up to three months), an approach that killed the host tissue and thereby limited viral detection to intact infectious particles. Other virus isolation methods that used co-cultured living host tissue⁸⁻¹⁰ may be more sensitive, enabling living tissue time to assemble viral particles in an environment ideal for viral growth. In addition, the robust neutralizing antibody titers detected in most birds also may have impacted the success of virus isolation attempts. All but one of the tissues found positive for WNV RNA by using the WN1 primer and probe set could not be confirmed by using the WN2 primer and probe set. This lack of confirmation was most likely caused by low levels of WNV RNA in the samples because nearly all of the positive

samples C_t scores were > 30 . In our assays, WN2 has been consistently less sensitive than WN1 in detecting WNV cDNA, and WN2 C_t scores are generally 2–4 C_t values higher than WN1. This reduced sensitivity may have contributed to the lack of confirmation.

The birds in our study were infected with WNV during the transmission season (June–September), housed in outdoor aviaries at ambient temperature and photoperiod over the winter months, and then tested for persistent WNV infection the following spring (March). The effect that day length, sex hormones, or other factors associated with changing seasons may have had on the persistence of WNV in avian hosts is unknown, but should be noted as potentially important. The use of different WNV genotypes showed that genotype or virulence phenotype did not appear to affect WNV RNA persistence; however, sample sizes were limited. Significant differences were not detected in the proportion of persistently infected birds when grouped by infecting genotype or magnitude of mean peak viremia. In addition, the frequency of WNV RNA persistence in naturally and experimentally infected House Sparrows was not significantly different. These data indicated that experimentally and naturally infected birds maintained WNV RNA in tissues over the winter period. These findings may, in part, help to explain the detection of low levels of WNV RNA identified in tissues from dead birds collected during winter and early spring before detection of virus in mosquitoes.^{13,34}

Our study demonstrated that WNV RNA could be detected in the tissues of persistently infected birds after an overwintering period. This finding supports the hypothesis that some avian hosts may develop long-term persistent WNV infections. However, it remains unresolved whether persistent WNV RNA can be attributed to infectious virus and whether persistently infected birds develop recrudescence viremias capable of restarting an avian–arthropod transmission cycle. Further work will be needed to characterize the temporal nature of persistence, identify mechanisms that lead to persistence, ascertain the cellular locations of persistent virus, and determine the infectivity of persistent infections to blood feeding mosquitoes.

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