Prevalence and Pathology of West Nile Virus in Naturally Infected House Sparrows, Western Nebraska, 2008

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Abstract. Nestling birds are rarely sampled in the field for most arboviruses, yet they may be important in arbovirus amplification cycles. We sampled both nestling and adult house sparrows (*Passer domesticus*) in western Nebraska for West Nile virus (WNV) or WNV-specific antibodies throughout the summer of 2008 and describe pathology in naturally infected nestlings. Across the summer, 4% of nestling house sparrows were WNV-positive; for the month of August alone, 12.3% were positive. Two WNV-positive nestlings exhibited encephalitis, splenomegaly, hepatic necrosis, nephrosis, and myocarditis. One nestling sparrow had large mural thrombi in the atria and ventricle and immunohistochemical staining of WNV antigen in multiple organs including the wall of the aorta and pulmonary artery; cardiac insufficiency thus may have been a cause of death. Adult house sparrows showed an overall seroprevalence of 13.8% that did not change significantly across the summer months. The WNV-positive nestlings and the majority of seropositive adults were detected within separate spatial clusters. Nestling birds, especially those reared late in the summer when WNV activity is typically greatest, may be important in virus amplification.

INTRODUCTION

Most field surveys of bird-associated arthropod-borne viruses (arboviruses) have sampled adult birds for the presence of virus or antibodies, perhaps because in many species adults can be sampled more easily and in greater numbers than can more dispersed and cryptic nestling birds. However, it is important to understand whether age influences birds' exposure to or relative involvement in arbovirus transmission, given potential differences in both the response to infection in adult versus nestling birds[1] and their ability to behaviorally avoid and/or to attract mosquito vectors.[2–4] Some general avian surveys reported that nestlings and immature birds were overrepresented in the virus-positive population,[5,6] and in one study peaks in West Nile virus (WNV) transmission were correlated with high seroprevalence and infection rates in hatching-year birds.[7] In contrast, other studies of WNV found relatively low seroprevalence in hatching-year birds in comparison to adults.[8,9]

Nestling birds may be more susceptible to viruses and more negatively affected by them than are adults of the same species. For example, WNV-infected young white leghorn chicks (*Gallus gallus domesticus*) exhibited greater viremias and more severe clinical response than older ones.[10] Western equine encephalomyelitis virus (WEEV) caused low to no mortality in adult house sparrows (*Passer domesticus*) in experimental infection studies[4,11] but is fatal to sparrow nestlings when they are infected naturally in the field[12] or experimentally infected in the laboratory.[4] Adult house sparrows inoculated with Buggy Creek virus (BCRV), an alphavirus in the WEEV antigenic complex,[13,14] develop transient to no viremia[15] and are not found viremic in field surveys (O'Brien V and Brown C, unpublished data), but field-sampled nestling house sparrows have high viremias and often die of virus infection.[16]

Although some information on WNV pathology is known for relatively young individuals of several raptor species,[17,18] we know almost nothing about the clinical pathology of arbo-viruses in nestling passerine birds,[16] possibly because nestlings or recently fledged passerines are rarely found dead or exhibiting clinical pathology attributable to arboviruses.[6,7,19] One study reported WNV RNA in kidney tissue of field-collected blue jay (*Cyanocitta cristata*) nestling carcasses, but necropsy was not performed on these birds.[20] Understanding WNV disease etiology in nestling birds may assist in explaining patterns of virus or antibody prevalence in field surveys and yield insight into ways that the virus may be transmitted.

A recent study of WNV in an urban area around Chicago, IL concluded that nestling passerine birds were not important in virus amplification: samples from 194 nestlings of 12 bird species yielded only one virus-positive and one antibody-positive individual.[21] However, the sampling was done in early summer before mosquitoes were abundant and before virus was commonly detected in vectors,[21] and thus not finding WNV in these nestlings is not surprising. A more complete understanding of the role of nestling birds in WNV amplification and transmission will require locating nests and sampling nestlings throughout a summer, including mid-to-late summer when WNV activity is most often detected,[7,9] and when late nesting, which is likely more common than generally assumed in North American birds,[22] is occurring.

The house sparrow historically has been heavily sampled for arboviruses, perhaps in part because of its abundance and peridomestic occurrence.[23] House sparrows were implicated in the transmission cycles of St. Louis encephalitis virus (SLEV)[24–26] and WEEV,[27] and they may serve as useful sentinels for SLEV activity.[28] House sparrows have previously been demonstrated to be competent hosts of WNV,[29,30] and studies in New York City during the initial outbreak concluded that sparrows likely contributed importantly to WNV transmission in that area in 1999.[31] A mid-summer roost of house sparrows in Colorado served as a prominent source of blood meals for *Culex tarsalis* Coquillett,[32] showing both behavioral (late-season aggregations of juveniles) and mosquito host-preference mechanisms for the involvement of house sparrows in WNV transmission.

As part of a study of the role of nestling birds in the transmission of BCRV,[16] we systematically sampled house sparrows of all ages in western Nebraska throughout the summer of 2008 and tested nestlings for WNV and adults for WNV-specific antibodies. This allowed us 1) to assess the potential

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role of this species and the different age classes in arbovirus transmission throughout the bird's breeding season, including in late summer at a time when some house sparrows are still nesting and when WNV should be most prevalent; 2) to describe pathology associated with WNV infection in nestlings; and 3) to study potential spatial and temporal differences in virus incidence. The northern Great Plains (Nebraska, South Dakota, North Dakota) have reported among the highest incidences of WNV cases of any region within the United States each year from 2003 through 2008,[33] and thus better understanding of WNV transmission dynamics in these relatively rural prairie areas is desirable.

MATERIALS AND METHODS

Study area and study species. Our study area, in western Nebraska along the North and South Platte rivers, was centered at the Cedar Point Biological Station (41°13′N, 101°39′W) in Keith County, and included portions of Garden, Lincoln, Deuel, and Morrill counties.[34,35] Because BCRV is associated exclusively with cliff swallow (*Petrochelidon pyrrhonota*) nesting colonies[35–37] and WNV samples were taken in the course of our work on BCRV, sampling of house sparrows in our study was restricted to those nesting in abandoned swallow nests on bridges and highway culverts, often in close proximity to human habitations. Cliff swallows are highly colonial passerine birds that build gourd-shaped mud nests on cliff faces, in highway or railroad culverts, and under bridges. The mud nests sometimes persist for many years after initial construction, particularly on man-made substrates. House sparrows evict cliff swallows from nests or occupy abandoned nests, usually at colonies near human activity, and will perennially use them until the nests fall from the substrate. House sparrows are semi-colonial, with colony size ranging from 1 to 20 active nests, and are highly sedentary, using the same breeding colony year after year.[38]

Sampling. House sparrow nestlings were sampled at 16 colony sites between May 29 and August 11, 2008. Each colony was visited from 1 to 4 times during the season to maximize coverage of first and subsequent broods produced at colony sites. Nestlings 4–17 days of age were removed from the nest, banded with U.S. Geological Survey numbered bands, and a 0.1 mL blood sample was taken by jugular venipuncture with a 29-gauge insulin syringe and placed in 0.4 mL of virus diluent.[39] Nestlings were then returned to the nest. Nestling age was estimated based on our experience with known-age house sparrow nestlings. Between one and six nests were sampled at each visit to a site, and each nestling was sampled only once. At one colony, two nestlings were found on the ground near an active nest, one dead about 5 m from the nest, and the other at the base of the nest, alive but showing clinical symptoms of disease (gasping, ruffled feathers, unresponsiveness, and lethargy). A blood sample was collected from the moribund nestling, and it died in hand. Both nestling carcasses were stored on wet ice and then at 4°C until shipment within 24 h to the National Wildlife Health Center (NWHC) for necropsy and virus isolation. Blood samples from nestlings were stored on wet ice in the field, returned to the laboratory, clarified by centrifugation, supernatant removed, and stored at −70°C until taken to the University of Tulsa for RNA extraction.

Adult and juvenile house sparrows were captured in mist nets at 14 colony sites every 5 to 7 days between May 25 and August 10, 2008. Birds were banded, sexed, bled as previously described for nestlings, and released. Blood samples were handled as for nestlings and shipped to the Center for Vectorborne Diseases Laboratory at the University of California-Davis (CVEC) for serological testing. All birds recaptured in the course of the season were re-sampled.

Human case data for WNV were obtained from the Nebraska Department of Health and Human Services (http://www.hhs.state.ne.us) website.

Virus detection. Viral RNA was extracted from house sparrow nestling sera by adding 25 μL sera in diluent to 100 μL of a guanidine thiocyanate-based lysis buffer. After the addition of 100 μL of 100% ethanol, RNA was extracted using the QIAmp Viral RNA Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. A positive control was included in each extraction, and negative controls were placed between every five samples. Extracted RNA samples were frozen at −20°C until shipped to CVEC for testing by real-time reverse transcription-polymerase chain reaction (RT-PCR) using primer and protocols described previously.[40,41] Blood samples were insufficient to also determine virus titers by plaque assay. In 2008, our TaqMan critical threshold values (Ct values) decreased significantly as a linear function of increasing virus concentration over a dilution series ranging from 1 to 5 $\log_{10}$ plaque forming units (PFU) of WNV per mL (Ct = 35.07–2.77 $\log_{10}$ PFU/mL; R^2 = 0.99, $P < 0.0001$). The Ct values of 13.1 and 36.9 would equate to virus titers of 7.9 to < 1.0 $\log_{10}$ PFU/mL.

To detect virus in tissue, portions of the spleen, brain, and liver were removed from the two nestling carcasses and virus isolation attempted. Each tissue was homogenized in a Stomacher 400 Circulator (Seward, Norfolk, UK) in 10 volumes of viral transport media.[42] The suspensions were centrifuged at $800 \times g$ for 30 min at 4°C, and 1 mL of the supernatant was inoculated onto Vero cell (ATCC CRL-1587) monolayers in 12 cm² flasks. The flasks were incubated at 37°C and 5% CO_2 and examined daily for cytopathic effects (CPE). Samples showing CPE were subjected to RT-PCR with WNV-specific primers to identify isolates as WNV.[43] Tissues also were tested for avian influenza using RT-PCR.[44]

Gross and microscopic pathology. At necropsy, body condition was scored, the carcasses were examined for external and internal pathology, and brain, spinal cord, liver, spleen, bursa, trachea, lung, heart, kidney, esophagus, proventriculus, ventriculus, pancreas, and intestine were collected for histopathology. Tissues were placed in 10% neutral buffered formalin, trimmed and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Immunohistochemical (IHC) staining on tissues from the sparrow that died in hand (Bird P10, Table 1) was performed at the Histology Laboratory, Department of Pathology, College of Veterinary Medicine, University of Georgia, following their protocols previously described.[45] Briefly, following deparaffinization, proteinase K was used for antigen retrieval and endogenous peroxidase was blocked using 3% hydrogen peroxide (H312-500, Fisher Scientific, Fair Lawn, NJ). Antigen retrieval was performed using Protease III, which is a mild enzymatic pretreatment (760-2020, Ventana Medical Systems, Inc., Tucson, AZ). Rabbit polyclonal anti-WNV antibody (80-015, BioReliance Corp., Rockville, MD) diluted 1:1000 using Dako Antibody Diluent (S0809, Dako, Carpinteria, CA) was applied to slides for 45 minutes, followed by biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories, Burlingame,

TABLE 1
West Nile virus-positive house sparrow nestlings, Morrill County, Nebraska, tested/collected on August 10, 2008*

Bird no.	Colony name	Location	Bird age (days)	Where found	Nest no.	Brood size	Virus identification (Ct score and/ or tissue)
316	CP	41° 30.869′ N 102° 38.971′ W	14	Fledged, hand caught	Likely 19	5	28.3
317	CP	41° 30.869′ N 102° 38.971′ W	14	In nest	19	5	16.5
319	CP	41° 30.869′ N 102° 38.971′ W	14	In nest	19	5	26.2
P10	CP	41° 30.869′ N 102° 38.971′ W	14	On ground, clinically ill	Likely 19	5	13.1/brain, spleen, liver
P11	CP	41° 30.869′ N 102° 38.971′ W	14	On ground, dead	Likely 19	5	Brain, spleen, liver
322	BC	41° 31.743′ N 102° 41.016′ W	12	In nest	14	4	36.9
333	CH	41° 34.106′ N 102° 46.576′ W	17	In nest	28	5	35.7

*Virus was identified by real-time reverse transcription-polymerase chain reaction (RT-PCR) (TaqMan Ct score) from sera, and virus isolation in tissue was confirmed with RT-PCR.

CA) and Streptavidin conjugated to horseradish peroxidase (Dako's LSAB 2; K1016, Dako). The substrate-chromogen system used was DAB (K3466, Dako) and slides were counterstained with Gills II hematoxylin and bluing. Positive tissue controls consisted of formalin fixed, paraffin-embedded heart from a WNV-infected raptor. As a negative control, the primary antibody was substituted with Universal Negative (N1699, Dako).

Liver was cultured for aerobic bacteria using 5% sheep blood and eosin-methylene-blue agars. Inoculated plates were incubated at 36°C for 48 h and bacterial isolates were identified using standard methods.

Serology. Sera were screened for antibodies against flaviviruses with an enzyme immunoassay (EIA) using a crude antigen prepared from Vero cell cultures of St. Louis encephalitis virus.[46] Positive EIAs had a ratio of the mean optical density of two antigen-positive wells divided by an antigen-negative well > 2.0. EIA positives were confirmed and identified with a 90% end-point plaque reduction neutralization test ($PRNT_{90}$) using the NY99 strain of WNV and the KERN217 strain of SLEV (70–80 PFU) on Vero cell culture. Twenty-seven of 28 EIA positives (96.4%) confirmed using $PRNT_{90}$ at a titer > 1:20 (3 EIA positives had too little sample for PRNT confirmation attempts). All positive samples were identified as WNV because the end-point titers were ≥ 4× the titer of the next most likely flavivirus (i.e., SLEV). Because greater than 95% of the samples EIA positive were confirmed by PRNT, and as others have reported that EIA is more sensitive than PRNT,[46,47] all samples that were EIA positive were considered to represent birds with prior exposure to WNV.

RESULTS

Virus detection. We tested 173 nestling house sparrows aged 4–17 days old from 53 nests for WNV. Mean overall age of those tested was 9.7 days (±0.3 SE). Seven nestlings (4%) were positive for WNV by RT-PCR and/or plaque assay. Older nestlings (≥ 12 days old) were more likely to be virus positive than younger ones (Wilcoxon two-sample test; Z = 2.63, P = 0.009) with mean age for virus-positive nestlings 14.1 days (±0.6 SE) and for virus-negative nestlings 9.6 days (±0.3 SE).

There was a strong temporal and spatial focus of WNV infection. All WNV-positive house sparrow nestlings were from three colonies 3 to 12 km apart along U.S. Highway 26 in Morrill County in the extreme western portion of the study area (Cluster A, Figure 1), and all virus detection occurred on August 10, 2008. Five of the seven WNV-positive nestlings were from the same colony, perhaps from the same nest (Table 1). Of the total nestlings sampled in August across the study area

(N = 57), 12.3% were WNV positive, and of those sampled in August in Morrill County alone (N = 21), 33.3% were WNV positive.

West Nile virus was isolated from all tissues cultured from the two nestling carcasses (brain, liver, and spleen; Table 1). Screening tests on tracheal and cloacal swabs from these two birds were negative for avian influenza. There was no evidence of bacterial infections grossly or microscopically, and only a few colonies of contaminant bacteria were isolated from the liver.

Gross and microscopic pathology. The two necropsied WNV-positive nestlings were in good body condition. There was a moderate amount of fecal material on the feet and ventral feathers of both birds, suggesting that they had been recumbent and sitting in feces. The spleens were approximately four times normal size. The bursae were very small, and the lobes of thymus were too small to be identified. The kidneys were enlarged beyond the renal crypts and tan rather than the normal deep red color. Histopathology was similar in both birds, although the bird found already dead had confounding autolysis.

For the initially moribund nestling (Bird P10, Table 1), the heart had large mural thrombi in the left ventricular apex and at the base of the left atria (Figure 2A). These organized thrombi effaced the endothelium and contained fibrin, necrotic cell debris, and heterophils. A small accumulation of heterophils was also adhered to the left atrioventricular heart valve. The thrombi were IHC negative for WNV antigen, but adjacent endocardium was positive. Moderate necrotic foci in the atrea were IHC positive. Positive IHC-WNV staining was also present in cells in the intermyofiber spaces, epicardium, the endocardium of the atria, ventricles, and heart valves, as well as the vascular endothelium.

The liver had severe, multifocal to coalescing acute hepatic necrosis without associated inflammation (Figure 2B). The distribution of necrosis was random without affinity for portal or central zones. No bacteria were seen in the liver, and none was isolated. Kupffer cells lining sinusoids stained more intensely for WNV antigen with IHC than hepatocytes in necrotic areas (Figure 3B). A small amount of hemosiderin pigment was present in hepatocytes and Kupffer cells.

The tunica intima and tunica media of both the aorta (Figure 3A) and the pulmonary artery had relatively large areas of multifocal IHC-positive WNV antigen. The areas of IHC-positive staining also defined the disruption of fibers in the tunica media of these arteries that was not obvious in the hematoxylin-eosin (HE)-stained sections. Multifocal areas of lung lost distinguishable alveolar architecture, and the interstitium was widened with edema, vacuolation, and a light infiltrate of heterophils and mononuclear cells. These regions
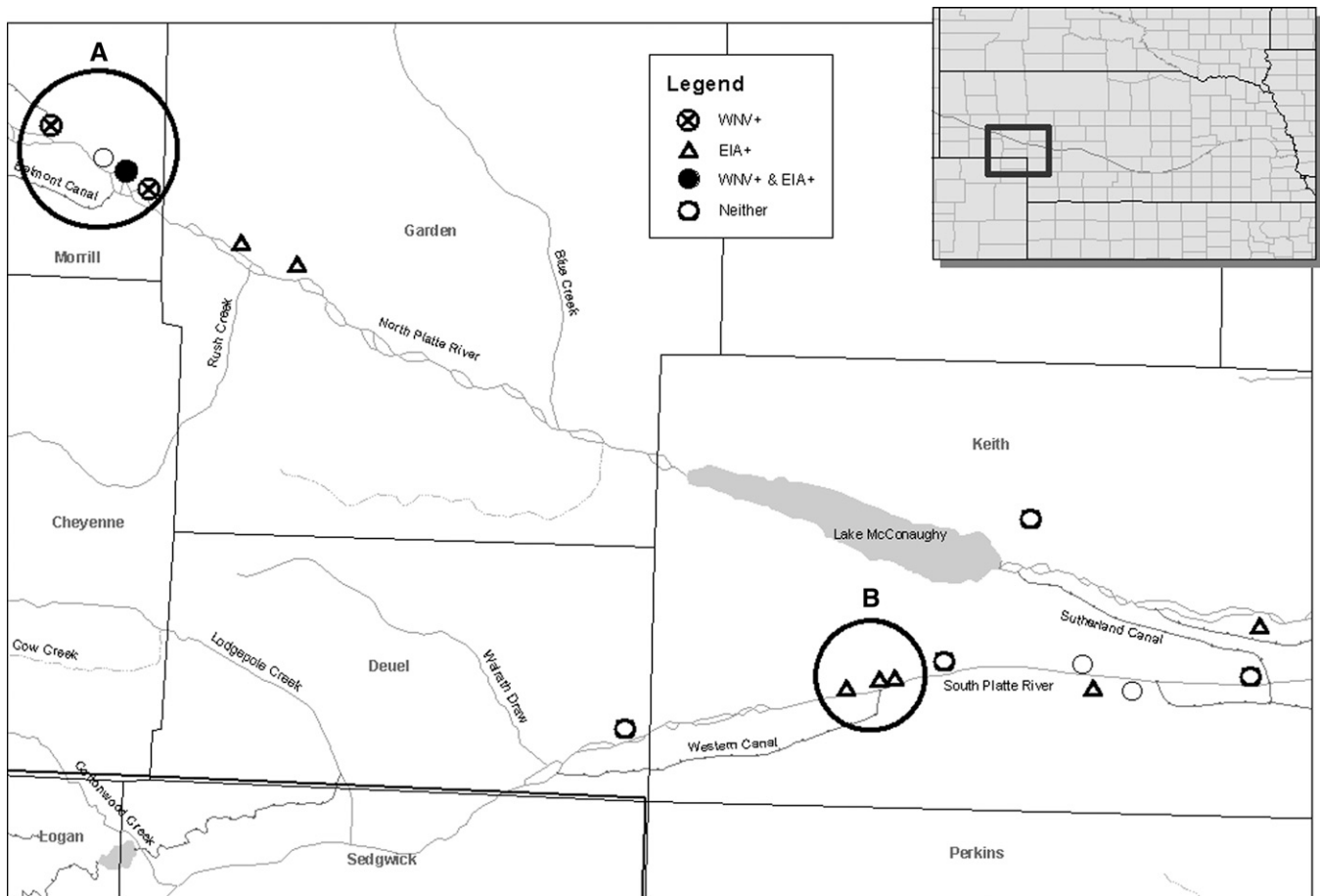
FIGURE 1. Locations of house sparrow colonies sampled for West Nile virus (WNV) in nestlings and antibodies in adults, May 29–August 11, 2008, western Nebraska. (**A**) Clusters of WNV amplification and (**B**) highest seroprevalence are circled. The colony with the greatest number of WNV-positive nestlings (CP) is the southeasternmost site in Cluster A.

of the lung subsequently stained intensely with WNV IHC (Figure 3C).

Vessels in the brain had multifocal swelling and vacuolation of endothelial cells with rare lymphocytes and plasma cells traversing vessel walls and forming loose accumulations in the perivascular space. There was regionally extensive IHC-WNV antigen staining of neurons and glial cells (Figure 3D) even in areas without detectible pathology or inflammation with HE stains. Clusters of Purkinje cells and scattered cells in the associated granular and molecular layers of the cerebellum were IHC positive (inset: Figure 3D). Inflammation could not be confirmed in the small sections of spinal cord; cut artifacts complicated diagnostic evaluation of spinal cord tissue and IHC staining was not performed.

Multifocal regions of kidney had acute tubular necrosis with casts of necrotic cells, protein, and urate material in the lumen; inflammation was minimal. Positive IHC staining was associated with necrotic tubules, multifocal clusters of renal interstitial cells, and occasional glomeruli. The bursa was markedly atrophied with only multifocal IHC-positive follicles. Staining in these bursal follicles was limited to the remnant medullary cells and epithelial cells. The cortical cells did not stain. The spleen was congested with a moderate accumulation of hemosiderin pigment. Spleen and skin were lost during sectioning, preventing IHC testing of these tissues. Trachea, esophagus, ventriculus, and proventriculus were negative for WNV IHC.

The pancreas and intestine were too autolyzed for diagnostic evaluation.

Serology. A total of 167 adult and juvenile house sparrows were captured, with 25 birds captured ≥ 2 times, resulting in 194 samples tested for antibodies to WNV. Of these, 31 samples were positive by EIA. No recaptured birds seroconverted between first and subsequent captures. Five birds were seropositive on both first and second capture. Overall, seroprevalence across all dates and all sites was 13.8% ($N = 189$; the birds seropositive on both captures were considered only once in this total). All hatching-year (fledged juvenile) house sparrows sampled ($N = 12$) were seronegative.

House sparrows with antibodies to WNV were found at eight colony sites across the study area (Figure 1). There appeared to be a spatial cluster of seropositive birds at three colonies between 0.22 and 4.98 km apart (Cluster B, Figure 1). Two colonies were located directly adjacent to a farmyard with large numbers of livestock, including domestic geese and chickens, and the other site was 0.3 km from an active agricultural operation. Seropositivity was higher in these three colonies (27.1%, $N = 61$) than in all others combined (11.3%, $N = 128$; $\chi^2_1 = 4.33$, $P = 0.037$).

When examining temporal seroprevalence, we excluded second capture data on birds that were EIA positive on both first and second capture, and used only the date of first capture for these individuals in our analysis. There was no increase in
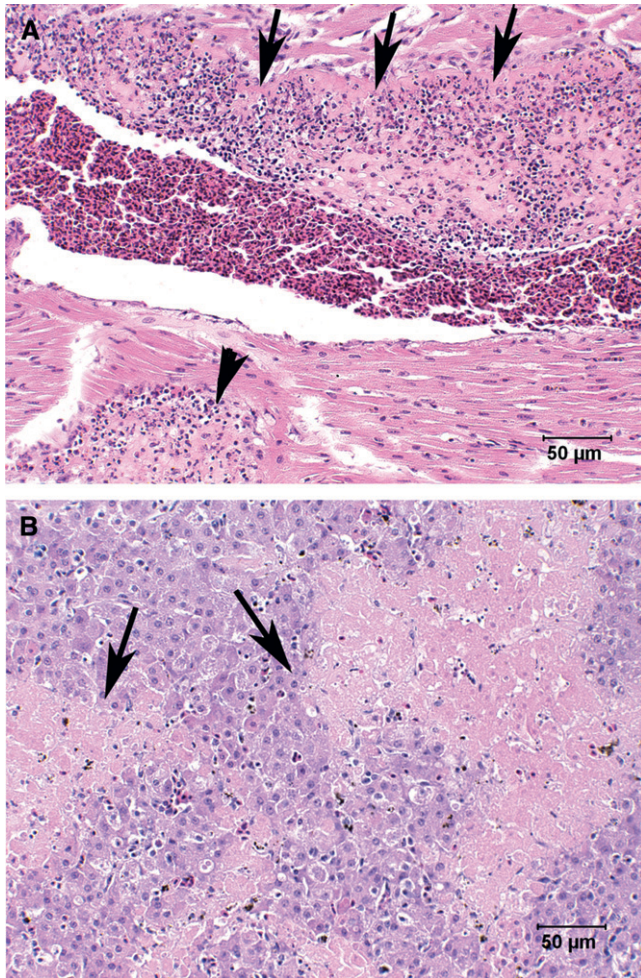
FIGURE 2. Hematoxylin and eosin stained heart and liver sections from a 14-day-old moribund house sparrow that died during handling as a result of natural infection with West Nile virus (WNV) (Bird P10; Table 1). (**A**) Heart with mural thrombus adhered to the left ventricular endocardium; loss of detectible endocardium associated with this mature thrombus (arrows). A subendocardial thrombus is also present (arrowhead). (**B**) Liver with acute multifocal to coalescing regions of necrosis (arrows) without inflammation.

seropositivity in the study area as the season progressed, as demonstrated by no significant difference in seroprevalence between the three months of May, June, and July ($\chi^2_2 = 0.12$, $P = 0.94$). Prevalence of antibodies also remained the same in the clustered colonies (Cluster B, Figure 1) from May to July at 18.5%, 21.4%, and 23.8%, respectively ($\chi^2_2 = 0.20$, $P = 0.90$).

DISCUSSION

Our systematic sampling of house sparrows throughout summer 2008 resulted in seven detections of WNV in nestling birds, all in mid-August near the end of the house sparrow's reproductive season. Across the summer, only 4% of nestlings sampled were virus positive, but when only the month of August was considered, 12.3% were positive. These results show that nestling house sparrows were relatively frequently infected with WNV in rural western Nebraska in late summer 2008. We found WNV in nestling birds only in August, a time when WNV activity in mosquitoes tends to peak at most locations.[7,9] This suggests that surveys of nestlings earlier in

the summer at times of low mosquito activity[21] are not likely to yield virus positives, even in areas where the virus may be endemic. Surveys throughout the summer are necessary to determine the full role of young birds in WNV amplification and transmission.

To our knowledge, this is the first description of WNV pathology with virus isolation from tissues of a naturally infected nestling passerine bird in North America. The IHC staining of WNV antigen in the lung, aorta, pulmonary artery, heart valves, endocardium, liver, brain, and kidney, with minimal inflammation, suggests that the terminal stage of viremia was rapidly fatal. The tropism WNV had for the endocardium, demonstrated by the IHC-staining cells lining the heart, was the likely initiator of the large thrombi in the left ventricle and left atria. It is reasonable to assume that the thrombi resulted in dramatic cardiac insufficiency. Hepatic necrosis was seen in both house sparrow nestlings, but was severe only in the nestling with the cardiac thrombi. Although the pattern of necrosis in the liver did not have a peri-central distribution, which would be expected in congestive heart failure, the relatively light staining of hepatocytes with WNV IHC (when compared with Kupffer cells) suggests that the severe hepatic necrosis seen was not due solely to WNV and that cardiac insufficiency may have exacerbated the necrosis. West Nile virus infection of the aorta and pulmonary arteries has not been previously reported, and infection may have compromised circulatory function.

The isolation of WNV from brain, liver, and spleen in the two field-collected nestlings, along with additional organs staining positively with WNV IHC, indicated extensive viral replication in these birds. Our findings agree with virus isolation from these same tissues in experimentally infected adult house sparrows[29] and other bird species.[48] The house sparrow nestlings in our study had pathology in multiple organs. Interestingly, although there is typically little pathology in corvids infected with WNV, virus is frequently isolated from multiple tissues.[48,49] This may reflect species differences in susceptibility to the virus and a rapid course of disease leading to death in crows and jays. The tissue pathology shown in the two field-collected house sparrows indicates that these nestlings likely maintained high viremic titers in blood, perhaps for several days before death, and presumably lived longer after infection than do corvids.

The lack of an increase in antibody prevalence across the 2008 season both for the study area as a whole and within a cluster of colonies with 27% seroprevalence (Cluster B, Figure 1), together with the relatively high percentage of house sparrows positive for antibodies to WNV, indicates that the area may have been involved in intensive WNV amplification in a preceding year (perhaps 2007) but not in early season 2008. High adult seroprevalence in May might signal prior-year exposure,[50] with WNV antibodies known to persist in house sparrows for up to 3 years.[51]

In contrast, our finding of WNV-positive house sparrow nestlings at colonies in Morrill County showed a potential zone of WNV amplification in 2008 that was farther to the west than in earlier years. Morrill County is sparsely populated, but still reported two human cases of WNV in 2008. Seroprevalence among adult house sparrows in the same Morrill County colonies in 2008 was only 4.5%. Although the sample size for adults there was small ($N = 22$), the low seroprevalence in 2008 is consistent with that area having minimal WNV activity
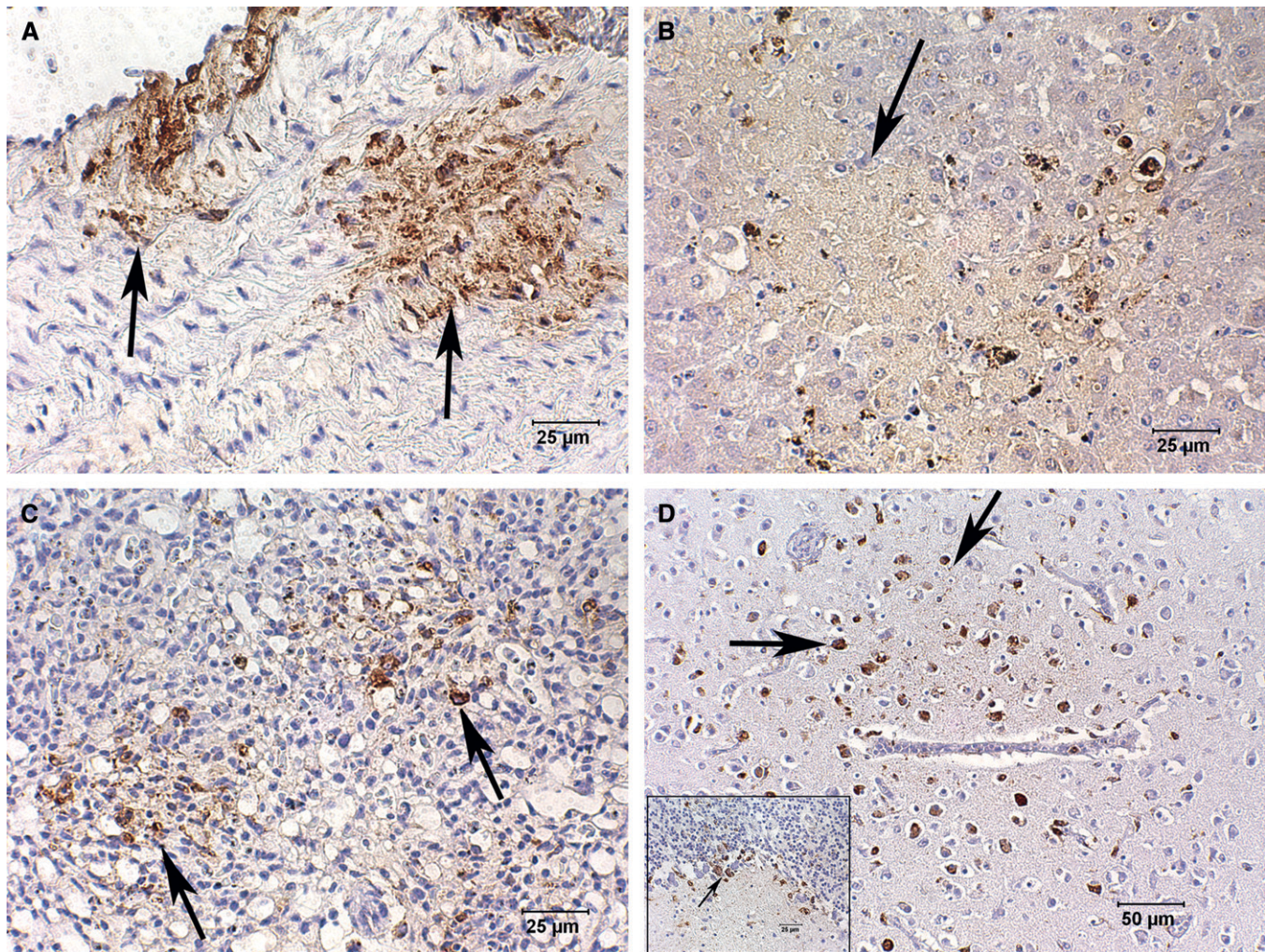
FIGURE 3.   West Nile virus (WNV) immunohistochemical (IHC) staining of tissues from the same house sparrow as in Figure 2 using Dako K3466, Dako Corp. DAB brown chromogen and counterstained with Gills II hematoxylin and bluing. (**A**) Aorta with positive IHC for WNV antigen in the tunica intima and tunica media (arrows). Disruption of fibers can be seen in the regions of positive staining. (**B**) Liver with faint WNV antigen staining of necrotic areas (arrow) and more intense staining of Kupffer cells at the margin of necrotic regions. (**C**) Multifocal areas of lung have lost alveolar architecture (arrows) and stain positively for WNV antigen. (**D**) Cerebrum with focus of intensely stained WNV antigen-positive glial cells and neurons (arrows). (**D**) Inset: cerebellum with WNV antigen staining of necrotic Purkinje cells (arrow) and surrounding glial cells associated with necrosis in the molecular layer and granular layer.

in preceding years. House sparrows are relatively sedentary during and between nesting seasons,[38] and consequently serology data from one year to the next may reflect localization of virus transmission. A caveat, however, is that adult house sparrows are known to succumb to WNV infection with variable fatality rates when experimentally infected,[29,30,51] so adult mortality cannot be ruled out as a cause of low seroprevalence in an area (e.g., Morrill County in 2008).

Our data showing different spatial clusters of WNV transmission between years match those from other regions that showed transmission occurring in discrete temporal and spatial foci, with some areas maintaining high infection rates between years[9] and others varying widely in both spatial and temporal parameters.[21,52] Highly focal WNV transmission was documented in Florida, with 78% of virus isolations from mosquitoes coming from 1 of 5 sites monitored in an area where WNV transmission had previously occurred.[53]

The house sparrows we studied were in nesting aggregations brought about by their use of cliff swallow nesting colonies, and thus they were perhaps more likely to be exposed to mosquitoes (and therefore to WNV) than sparrows nesting in a more dispersed distribution. Groups of birds can be highly attractive to mosquitoes: Brown and Sethi[54] found a positive relationship between cliff swallow nesting colony size and local mosquito abundance, with the more ornithophilic species recruited to the vicinity of the birds' nests. In a study of California birds, the house finch (*Carpodacus mexicanus*) was frequently found seropositive and at high risk for WNV infection,[55] perhaps because this species often nests in low-lying vegetation in loose aggregations that may increase its exposure to mosquitoes. Clusters of American crows (*Corvus brachyrhynchus*) have been linked to increased infection rates in *Culex pipiens quinquefasciatus* Say mosquitoes in California,[56] and crow roosting habits may also promote bird-to-bird transmission of WNV.[57]

In one colony (CP; Figure 1), all five WNV-positive nestlings may have been from the same nest (Table 1). All appeared to be the same age (14 days old) and about ready to fledge,

whether found in the nest or on the ground. There were two known active nests in the colony at the time, and the other nest contained 7-day-old nestlings that were virus negative. The presence of five, near-adult size birds occupying a single nest may be highly attractive to host-seeking ornithophilic mosquitoes: as brooding decreases and nestlings become larger and thus more exposed to mosquito attack, feeding on nestlings by mosquitoes may increase.[4,58] Furthermore, the crowding experienced by older and larger nestlings before fledging, the high titer and duration of viremias documented in nestlings infected with arboviruses,[1,10] and the decreased nest sanitization by house sparrows in the days immediately before fledging of young[23,59] could lead to an increased likelihood of bird-to-bird transmission of WNV.[60] In one study, contact transmission rates of WNV through oral and cloacal shedding were as high as mosquito-induced transmission in some corvids.[29] House sparrow nestlings testing positive for WEEV in a Texas survey tended to be clustered by nest, with virus concurrently detected in oral sampling, which indicates possible contact transmission of WEEV between birds in nests.[12] Direct transmission of BCRV was demonstrated in an experimental inoculation study involving house sparrows,[15] perhaps through fecal contamination of water or food sources. The two nestlings examined by necropsy had feces on the ventral body surface, indicating they may have become unable to preen or perhaps to properly defecate out of the nest entrance because of morbidity from virus infection, which could further expose nest mates to WNV.

The finding of WNV-positive house sparrow nestlings in late summer illustrates the importance of continued research into the potential contribution of nestling birds to arbovirus transmission cycles, especially among late nesting individuals. The clustering of WNV in these birds also suggests that virus infection may occur in spatially discrete foci even in areas where it is generally common and means that widespread sampling across a landscape may sometimes be necessary to detect focal hotspots.

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