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Experimental West Nile Virus Infection in Eastern Screech Owls (*Megascops asio*)

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SUMMARY. Eastern Screech Owls (EASOs) were experimentally infected with the pathogenic New York 1999 strain of West Nile virus (WNV) by subcutaneous injection or *per os*. Two of nine subcutaneously inoculated birds died or were euthanatized on 8 or 9 days postinfection (DPI) after <24 hr of lethargy and recumbency. All subcutaneously inoculated birds developed levels of viremia that are likely infectious to mosquitoes, with peak viremia levels ranging from $10^{5.0}$ to $10^{9.6}$ plaque-forming units/ml. Despite the viremia, the remaining seven birds did not display signs of illness. All birds alive beyond 5 DPI seroconverted, although the morbid birds demonstrated significantly lower antibody titers than the clinically normal birds. Cagemates of infected birds did not become infected. One of five orally exposed EASOs became viremic and seroconverted, whereas WNV infection in the remaining four birds was not evident. All infected birds shed virus *via* the oral and cloacal route. Early during infection, WNV targeted skin, spleen, esophagus, and skeletal muscle. The two morbid owls had myocardial and skeletal muscle necrosis and mild encephalitis and nephritis, whereas some of the clinically healthy birds that were sacrificed on 14 DPI had myocardial arteritis and renal phlebitis. WNV is a significant pathogen of EASOs, causing pathologic lesions with varying clinical outcomes.

RESUMEN. Infección experimental con el virus del oeste del Nilo en un tecolote oriental (*Megascops asio*).

Mediante inyección subcutánea o por la vía oral, se infectaron experimentalmente tecolotes orientales con la cepa patógena (New York 1999) del virus del oeste del Nilo. Dos de nueve aves inoculadas subcutáneamente murieron o fueron sacrificadas 8 o 9 días posteriores a la infección después de menos de 24 horas de letargia y recumbencia. Todas las aves inoculadas subcutáneamente desarrollaron niveles de viremia considerados infecciosos para mosquitos, con picos de viremia en el rango de $10^{5.0}$ a $10^{9.6}$ unidades formadoras de placa por ml. A pesar de la viremia, las 7 aves restantes no desarrollaron signos de la enfermedad. Todas las aves vivas resultaron serológicamente positivas 5 días posteriores a la inoculación, sin embargo, las aves enfermas mostraron títulos de anticuerpos significativamente menores que las aves clínicamente normales. Las aves alojadas en la misma jaula de las aves infectadas no se infectaron. Uno de cinco tecolotes orientales expuestos por vía oral entró en viremia y fue serológicamente positivo, mientras que en el resto de los animales la infección con el virus del oeste del Nilo no fue evidente. Todas las aves infectadas diseminaron el virus por la ruta oral y cloacal. En los estados iniciales de la infección el virus del oeste del Nilo afectó la piel, el bazo, el esófago y el músculo esquelético. Los dos tecolotes enfermos mostraron necrosis del músculo esquelético y cardíaco, así como encefalitis y nefritis leves, mientras algunas de las aves clínicamente sanas que fueron sacrificadas 14 días posteriores a la infección, mostraron arteritis miocardial y flebitis renal. El virus del oeste del Nilo es un patógeno de importancia en los tecolotes orientales, causando lesiones patológicas con consecuencias clínicas variables.

Key words: West Nile virus, Eastern Screech Owl, *Megascops asio*, experimental infection, viremia, shedding, antibody, histopathology

Abbreviations: BA1 = bovine albumin-1; DPI = days postinfection; DPIE = days postinfection of euthanasia/death; DPS = days postinfection of peak shedding; EASO = Eastern Screech Owl; H&E = hematoxylin and eosin; IHC = immunohistochemistry; IR = inoculation route; MNL = mononuclear leukocyte; MPS = mean peak shedding titer; PFU = plaque-forming units; PRNT = plaque reduction neutralization test; $PRNT_{90}$ = endpoint 90% neutralization level; RT-PCR = reverse transcriptase polymerase chain reaction; RDS = range of days postinfection of shedding; SC = subcutaneous; SLEV = St. Louis encephalitis virus; WNV = West Nile virus

Raptors, including owls, have been reported to experience morbidity and mortality because of infection with West Nile virus (WNV; genus *Flavivirus*, family *Flaviviridae*) (6,7). Past experimental work with WNV and raptors has not resulted in overt disease, although it demonstrated that several raptor species can serve as competent amplifying hosts (9,13). The susceptibility of different raptor species, including acute and chronic response to WNV infection, remains poorly understood. Although natural WNV infection in raptors (4,6,7,14,16,17) has been documented, reports of cases with known infection histories in raptors are sparse (9,13).

The rate of clinical WNV cases in some raptor species at breeding facilities and rehabilitation centers in North America has been high (6,7,16,17), but little information is available as to the pathogenesis of WNV infection in these birds. Among native North American owl species, great horned owls (*Bubo virginianus*) develop high-level viremia following infection with WNV and are likely a competent reservoir host (9,13). Eastern Screech Owls (EASOs; *Megascops asio*) are abundant in WNV endemic areas of North America east of the Rocky Mountains and extending from southern Canada to Mexico. To evaluate the hypothesis that EASOs are competent hosts for WNV, we performed experimental infections and report clinical observations, viremia, oral and cloacal shedding, virus isolation from tissues, detection of viral RNA in feathers, antibody titers, and micro- and macroscopic lesions that resulted from these infections.

MATERIALS AND METHODS

Animals and experimental design. Ten juvenile EASOs between 5 and 8 months of age were obtained from a captive breeding colony at Patuxent Wildlife Research Center and housed in free-flight rooms in a Biosafety level-3 facility at Colorado State University. Birds were given approximately 2 wk to adjust to their new environment,

during which time they were screened for antibodies to WNV and St. Louis encephalitis virus (SLEV). The owls were fed frozen-thawed mice *ad libitum* and were weighed every 3 or 4 days, with daily food consumption recorded by weight. After infection, they were evaluated daily for clinical signs.

In the initial experiment, five birds were inoculated subcutaneously over the breast with 1000–2000 plaque-forming units (PFU) of the NY99-4132 strain of WNV (3,11), which was originally isolated from an infected crow and passaged once in Vero cells, once in C6/36 mosquito cells, and once in baby hamster kidney-21 cells. Each of these five EASOs was housed in a separate pet carrier (70 × 55 × 60 cm) along with a noninoculated cagemate. Blood (0.2 ml) was collected daily from 1 to 5 days postinfection (DPI), on 7 DPI, and on the day of death or euthanasia. Blood was expelled into 0.9 ml of bovine albumin-1 (BA1) medium (Hanks M-199 salts, 1% bovine serum albumin, 350 mg/liter sodium bicarbonate, 100 units/ml penicillin, 100 mg/liter streptomycin, 1 mg/liter amphotericin B in 0.05 M Tris, pH 7.6), allowed to clot for 15–30 min at room temperature, centrifuged (6000 × *g* for 5 min), and frozen at −80 C as the equivalent of 10% serum until assayed for virus. Oral and cloacal swabs were obtained daily from each of the 10 birds from 1 to 14 DPI unless euthanatized earlier. Swabs were broken off in tubes containing 1 ml of BA1 medium and frozen at −80 C within 15 min of sampling.

Because of the difficulty of obtaining these birds, those that failed to become detectably viremic and failed to seroconvert (at 2 and 8 wk from last contact with infected owls) to WNV were reused in subsequent experiments. All of the contact control birds from the first experiment were challenged by feeding one whole head from WNV-infected mice 8 wk after last contact with infected EASOs. The mice had been inoculated at approximately 8 wk of age by intraperitoneal inoculation of $10^{3.8}$ PFU (NY99-4132 strain) and euthanatized when clinical signs became evident (7–9 DPI). The head of one mouse from this group was homogenized with a Tenbroeck grinder, and virus titration indicated that it contained approximately $10^{6.8}$ PFU of WNV. EASOs fed infected mice were bled 1–5, 7 and 10 DPI for virus isolation, and oral and cloacal swabs were collected daily from 1 to 14 DPI.

Four of the five birds fed infected mice failed to become infected, as indicated by failure to detect virus in blood or swabs and by lack of seroconversion by 3 wk postexposure. These birds were therefore inoculated subcutaneously with 1000–2000 PFU WNV as in the first experiment 24 days after oral exposure to infectious mouse heads. Blood samples and swabs from oral and cloacal cavities were collected daily until 5 DPI, at which time the birds were euthanatized and necropsies performed.

Necropsy, tissue collection, and pathologic evaluation. Birds that were clinically affected or scheduled for euthanasia were euthanatized by sodium pentobarbital overdose, and necropsies were performed within 2 hr of death. Gross findings were noted, and the following tissues were collected for virus isolation (approximately 0.5 cm^3) and placed in 1 ml of BA1 in cryovials: heart, lung, liver, spleen, kidney, small intestine, skin, gonads, esophagus, cerebrum, eye, and skeletal muscle. Tissues were homogenized in a mixer mill (5 min at 25 cycles/sec; Retsch GmbH, Haan, Germany) in 1 ml of BA1 medium containing 20% fetal bovine serum, and homogenates were clarified by centrifugation (12,000 × *g* for 3 min). Supernatants were stored at −80 C and later tested for virus by plaque assay.

Samples of heart, lung, liver, spleen, kidney, skeletal pectoral muscle (not corresponding to inoculation site), proventriculus, ventriculus, duodenum, pancreas, midjejunum, large intestine, cecum, cerebrum, cerebellum, midbrain, and spinal cord (~1.0-cm section from the proximal cervical region) were fixed in 10% neutral buffered formalin for histopathologic evaluation. The tissues were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Apparent mineralization in tissues was confirmed by von Kossa staining (8).

Histologic lesion grading was based on the level of mononuclear leukocyte (MNL, lymphocytes and plasma cells) infiltration and tissue involvement and ranged from mild (1–4 foci of MNL infiltration [or microgliosis in the case of brain tissue]) to moderate (5–11 foci of MNL infiltration or microgliosis) to severe (extensive [>30% section] inflammation or microgliosis). Hepatic MNL infiltration patterns were difficult to score because of lack of negative control tissues; therefore, nonspecific levels of background infiltration were not considered. Observations of mineralization, vasculitis, and necrotizing arteritis were not graded but were analyzed on a qualitative basis.

Virus isolation and antibody assays. Virus isolation and titration was performed on serum samples, oral and cloacal swabs, and tissue homogenates by plaque assay on Vero cells as previously described (3). Minimum levels of WNV detection by virus isolation were 1.7 log PFU/ml for blood, 0.7 log PFU/ml for swabs, and 0.7 log PFU/0.5 cm^3 for tissue. Virus isolation from all WNV-positive tissues was confirmed through reisolation. Neutralizing antibody titers were determined by plaque reduction neutralization test (PRNT) (2) with Vero cells in six-well plates. Serum samples (7 DPI) and terminal serum samples were titrated together for comparison. The challenge virus for WNV PRNT was the same as used for inoculation of birds, and strain TBH-28 was used for the SLEV PRNT. Sera were heat-inactivated (56 C for 30 min) before being assayed by PRNT, and titers were expressed as the reciprocal of the highest serum dilution showing greater than 90% reduction ($PRNT_{90}$) compared with control wells.

Detection of WNV in feathers. One breast feather was removed from EASOs euthanatized on 5 DPI immediately following euthanasia. For all other EASOs, feathers were removed on 7 DPI and on the day of death or euthanasia (8, 9, or 14 DPI). Feathers were ground in a Tenbroeck tissue grinder with 1 ml of BA1 and centrifuged at 12,000 × *g* for 3 min. TaqMan reverse transcriptase polymerase chain reaction (RT-PCR) methods for detection of WNV RNA were performed as previously described (10), except for the use of Qiagen's viral RNA minikit (QIAGEN Inc., Valencia, CA) for RNA extraction and the Icycler IQ™ Real-time Detection system (Bio-Rad, Hercules, CA) for the 45 amplification cycles.

RESULTS

Clinical signs, viremia, and viral shedding. None of the five contact control birds became viremic or developed antibodies by 8 wk after last contact with the infected cagemate and were therefore subsequently used in an oral exposure experiment. Three of the WNV-inoculated birds failed to develop discernible clinical signs and were euthanatized on 14 DPI. The other two subcutaneously inoculated EASOs showed clinical signs attributed to WNV infection, which included fluffed feathers, lethargy, and emaciation. The first bird began showing clinical signs on 8 DPI and died overnight, and the second was recumbent and lethargic on 9 DPI and was euthanatized. These birds exhibited severe weight loss of 20%–21% body weight over the 6–8 days before death. Changes in food consumption as measured per cage (with one inoculated and one noninoculated EASO) were difficult to interpret because the amount eaten by each individual cagemate was indistinguishable. However, food amounts consumed in nonmorbid owl cages did not appear to differ over time from those in morbid owl cages, and these levels also did not appear to differ greatly from preinfection levels of food consumption.

Each of the five subcutaneously inoculated EASOs became viremic, with peak viral titers ranging from $10^{5.8}$ to $10^{9.6}$ PFU/ml serum. Four of the five birds were viremic by 1 DPI and remained viremic through 5–7 DPI. The two owls that became morbid had the highest peak viremias (Fig. 1). Subcutaneously inoculated EASOs began to shed WNV from the oral cavity between 2 and 3 DPI, and shedding persisted for up to 13 DPI. The peak level of oral shedding in an individual was $10^{6.3}$ PFU on 8 DPI. Cloacal shedding also began between 2 and 3 DPI, and the highest level was $10^{5.7}$ on 8 DPI. After 5 DPI, cloacal shedding from some subcutaneously inoculated birds was intermittent and lasted up to 11 DPI. Mean peak oral WNV shedding levels from morbid EASOs

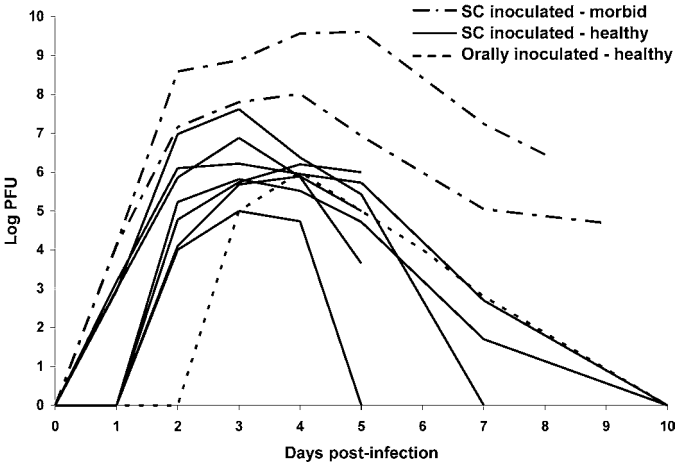


Fig. 1. Viremia in Eastern Screech Owls with experimental WNV infection. SC = subcutaneously.

Table 2. Viral titers in tissues of Eastern Screech Owls with experimental WNV infection.[A]

			WNV titer (log PFU/0.5 cm^3)											
EASO	IR	DPIE	Ce	Ki	He	Sp	Li	Lu	SI	Es	Go	Sk	Ey	Mu
1	SC	5	0	1.2	0	5.2	1.9	1.2	0	3.9	0	4.8	0	2.5
2	SC	5	0	2.2	2.5	4.7	1.4	3.3	3.0	5.1	2.5	4.9	2	4.9
3	SC	5	0	3.5	4.1	4.4	3.1	4.2	3.1	4.7	2.6	5.5	2.7	5.6
4	SC	5	0.7	5	3.7	4.8	3	5.6	0.7	5	4	6.9	2.1	5.8
5[B]	SC	8	5.1	7.3	7.9	5.6	5.1	7	4.7	6.8	4.7	6.6	5.9	8.8
6[B]	SC	9	3	6.2	7.4	5	1.2	5.1	2.1	6	NT	6.5	4.9	8
7	SC	14	0	2.1	0	1.2	0	0	0	0	0.7	0	2	0
8	SC	14	0	1.5	0	0	0	0	0	0	0	0.7	0.7	0
9	SC	14	0	0	0	0	0	0	0	0	0	0	1.3	0
10	O	14	0	1.5	0	0	0	0	0	0	0	0	1.2	0

[A]IR = inoculation route; Ce = cerebrum; Ki = kidney; He = heart; Sp = spleen; Li = liver; Lu = lung; SI = small intestine; Es = esophagus; Go = gonad; Sk = skin; Ey = eye; Mu = skeletal muscle; NT = not tested.
[B]EASOs exhibiting WNV-related morbidity.

were not significantly different from those of clinically normal birds euthanized on 14 DPI, although cloacal shedding was significantly greater for morbid birds (mean of $10^{5.6}$ *vs.* $10^{2.9}$, $P < 0.05$, *t*-test; Table 1).

None of the EASOs fed infectious mouse heads showed discernible clinical signs, but one became viremic, with an onset at 3 DPI and peak titer of $10^{6.0}$ PFU/ml serum on 4 DPI (Fig. 1). The quantity of WNV shed in oral secretions from this orally inoculated EASO was similar to that of subcutaneously inoculated birds, lasting for 5 days (6–10 DPI). Cloacal shedding for this bird was at relatively low levels and was intermittent between 3 and 12 DPI (Table 1).

The four owls that did not become infected by oral exposure were used in a final experiment and inoculated with WNV by subcutaneous injection. These birds were euthanized on 5 DPI to evaluate acute pathologic changes. Viremia and shedding profiles of these birds were similar to the EASOs in the initial group of subcutaneously inoculated birds that lacked clinical signs and were euthanized on 14 DPI (Fig. 1). Viremias first became detectable on 1–2 DPI. Peak viremia titers ranged from $10^{5.0}$ to $10^{6.9}$ PFU/ml serum and occurred on 3–4 DPI. Oral and cloacal shedding began on 3 DPI for most of these birds and lasted until euthanasia on 5 DPI in most cases (Table 1).

Virus isolation from tissues. Viral titers in tissues from all birds are presented in Table 2. In the initial experiment, WNV was isolated in relatively high titer from all tissues examined from the two subcutaneously inoculated EASOs that became sick or died at 8 or 9 DPI (EASOs 5, 6). For the three subcutaneously inoculated owls from the initial experiment that appeared healthy when euthanized on 14 DPI (EASOs 7–9), WNV was isolated from kidney, spleen, gonad, skin, and eye. The orally inoculated owl (EASO 10) that became viremic had low levels of WNV isolated from kidney and eye after euthanasia on 14 DPI. Among the EASOs in the final experiment that were euthanized on 5 DPI (EASOs 1–4), virus was isolated from most tissues with the exception of cerebrum, from which only one of the four birds had a low level of WNV.

Table 1. Viral titers of oral and cloacal swabs from Eastern Screech Owls with experimental WNV infection.[A]

				WNV titer (log PFU/ml)					
				Oral			Cloacal		
Infection route	*n*	Health status	DPIE	MPS	DPS	RDS	MPS	DPS	RDS
SC	4[B]	Healthy	5	3.3	4–5	3–5	1.8	3–5	3–5[C]
SC	2	Morbid	8–9	6.02	8	2–D	5.6	6–8	3–D
SC	3	Healthy	14	5.3	6–8	2–13[C]	2.9	5–6	2–11[C]
O	1	Healthy	14	6.02	7	6–10	2.3	7	3–12[C]

[A]DPIE = days postinoculation euthanasia/death; MPS = mean peak shedding titer; DPS = days postinfection of peak shedding; RDS = range of days postinfection of shedding; SC = subcutaneous; O = oral; D = day bird died or was euthanized because of clinical signs (this day was between 8 and 9 DPI).
[B]Euthanized on 5 DPI, so levels shown might not reflect actual peak or ranges of shedding.
[C]Shedding was intermittent within the given range.

Direct contact transmission. WNV transmission failed to occur among cagemates in all five cases, as evidenced by failure to detect viremia and lack of neutralizing antibodies 2 and 8 wk after the end of the period of contact with infected cagemates. Noninfected birds were in close contact with infected owls (often observed perched within centimeters of each other) that were shedding relatively high levels of virus in both oral and cloacal secretions (Table 1).

Feather analysis. Feathers from three of five subcutaneously inoculated EASOs tested positive for WNV RNA on 7 DPI. The three EASOs with WNV-positive feathers were also those that developed the highest level of viremia. Two of these three owls became severely ill, and their feathers also tested positive on the day of death or euthanasia (8 and 9 DPI). For birds that became viremic but survived, all feathers tested on 14 DPI were negative for WNV RNA. Feathers from orally infected EASO tested negative on 7 and 14 DPI. From the later group of subcutaneously inoculated EASOs, feathers from all four birds tested negative on 5 DPI when birds were euthanized.

Serologic responses. At death or euthanasia, all EASOs that had become viremic had $PRNT_{90}$ antibody titers of WNV of ≥ 20, except for the four birds euthanized on 5 DPI, which had levels of <10. Among the five birds inoculated with WNV in the original experiment, $PRNT_{90}$ titers on 7 DPI ranged from 320 to 5120 for the three birds that failed to become ill and from 20 to 40 for the two birds that showed clinical signs. The three apparently healthy EASOs exhibited a fourfold or greater decrease (up to 16-fold) in WNV $PRNT_{90}$ between 7 and 14 DPI, which was also true for the orally inoculated EASO that became viremic. One clinically ill EASO exhibited an eightfold increase in $PRNT_{90}$ titer between 7 and 9 DPI, and the other exhibited a twofold decrease between 7 and 8 DPI.

Pathology. The subcutaneously inoculated EASO that died on 8 DPI was emaciated at necropsy, and the myocardium was diffusely pale. The EASO euthanatized on 9 DPI because of clinical illness had diffuse, severe skeletal muscle wasting evident in the pectoral muscles, as well as pale mottling at the apex and base of the heart (~3–4 mm). Gross lesions were not significant in the seven apparently clinically normal subcutaneously inoculated birds euthanatized on 5 or 14 DPI or in the orally inoculated EASO euthanatized 14 DPI, and all were in good body condition.

Histopathologic lesions in WNV-infected EASOs are summarized in Table 3. Lesions varied with clinical outcome and time of euthanasia. The two EASOs exhibiting clinical signs of illness had the most severe histologic lesions. Myocardial lesions in morbid EASOs included multifocal areas of necrosis, often with fine mineralization, MNL infiltration of the myocardium and epicardium, and mild to moderate hemorrhage (Figs. 2, 3). Both morbid EASOs had myocardial perivascular MNL accumulations, and in one bird, heterophils were prominent in some perivascular areas. Pectoral muscle lesions were characterized by diffuse, multifocal fiber necrosis with fine mineralization (Figs. 4, 5); other areas of skeletal muscle included moderate to severe MNL infiltration. Renal lesions included edema, interstitial infiltration with MNL and phlebitis of variable severity. One bird had moderate, diffuse hepatic vacuolar degeneration with necrosis of a small group of hepatocytes. Both birds had varying proportions of MNL and heterophilic perivascular accumulations; some MNLs were observed within hepatic venous walls, suggestive of phlebitis. One morbid EASO had mild MNL pancreatitis and the other had a focus of necrosis within the pancreas. Both birds had multifocal areas of mild gliosis within the cerebrum, cerebellum, or both; within the cerebellum, gliosis was in the molecular layer. No lesions were observed in the spinal cord of any EASOs in this study.

Each of the three subcutaneously inoculated EASOs that appeared clinically normal when euthanatized on 14 DPI had similar lesions, including mild MNL accumulations in the myocardium, kidney and skeletal muscle, and mild encephalitis. Unlike the EASOs that exhibited clinical signs of illness, these birds did not have myocardial necrosis; however, all three owls had varying degrees of myocardial arteritis (Fig. 6) with mild to moderate myocarditis. Affected myocardial vessels were only observed in the interventricular septum. Two of three owls had mild to moderate skeletal muscle MNL infiltration. All of these owls had mild to moderate focal MNL nephritis; two had perivascular MNLs with some inflammatory cells within venous walls, suggestive of phlebitis. All three owls had mild perivascular accumulations of MNLs in the liver, with some MNLs within the venous endothelium suggestive of hepatic phlebitis. Two owls had mild to moderate diffuse hepatic vacuolar degeneration. One owl had moderate duct-associated MNL infiltrations with macrophages in the pancreas. Two of three owls had mild focal nonsuppurative encephalitis characterized by microglial nodules in at least one brain compartment, including cerebrum, cerebellum (Fig. 7), and midbrain. In one of these birds, the microglial nodules appeared to be vessel associated and had accompanying karyorrhectic debris. Additionally, this bird had microhemorrhages in the cerebellar molecular layer.

The orally inoculated owl and the owls euthanatized on 5 DPI had minimal histologic lesions except for myocardial arteritis in the orally inoculated bird. All of these EASOs had multifocal MNL accumulations in the liver, mostly involving periportal and perivascular regions. Lesions in the orally inoculated EASO included mild focal myocarditis with necrotizing arteritis and mild MNL nephritis. One of the four subcutaneously inoculated owls euthanatized on 5 DPI had mild, focal MNL myocarditis. All four owls had mild interstitial nephritis; one had mild myositis and another had mild focal microgliosis in the cerebellar molecular layer.

Table 3. Degree and characterization of histopathologic lesions in tissues from Eastern Screech Owls with experimental WNV infection.[A]

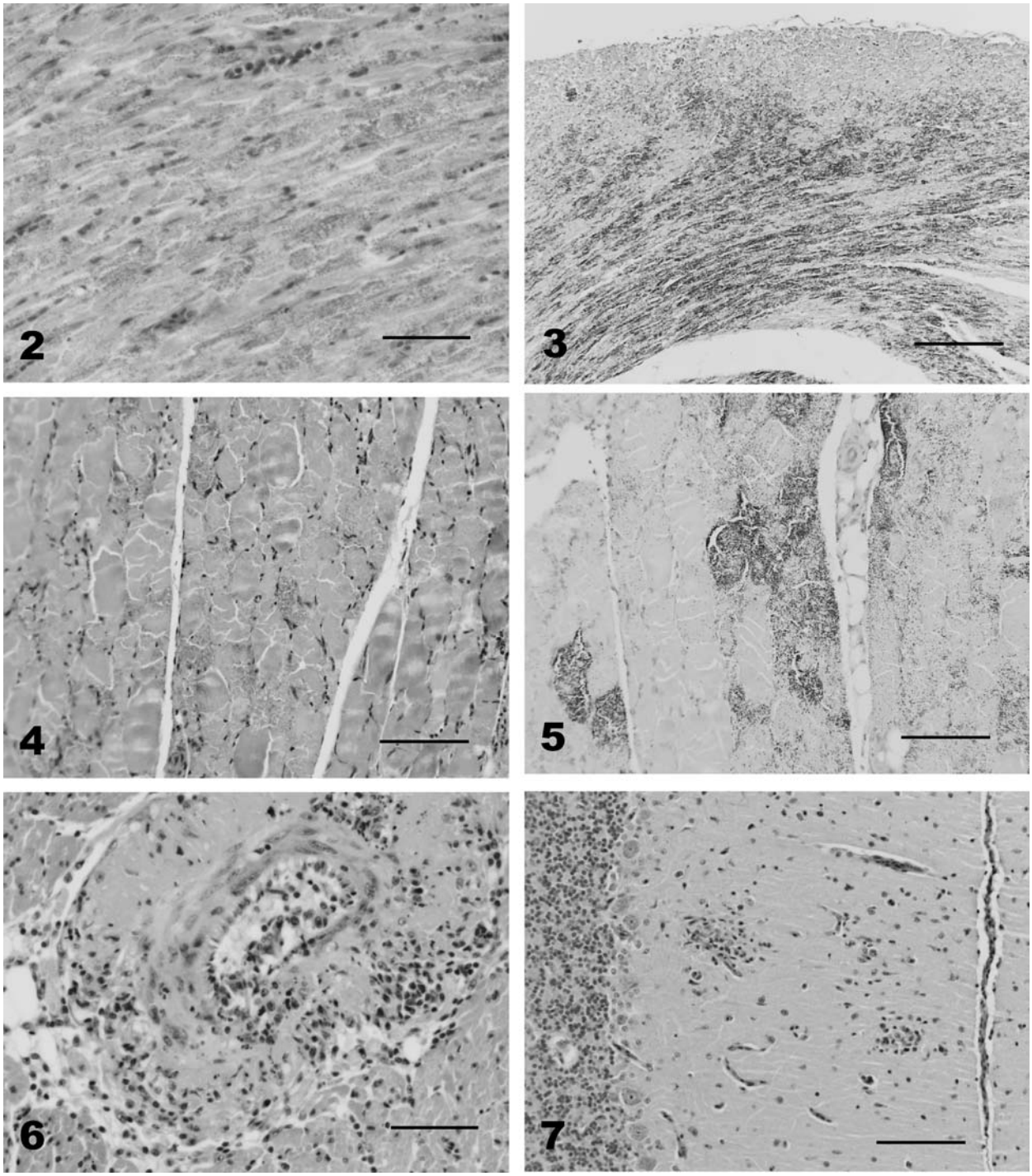
			MNL infiltration in parenchyma of tissue[B]								Other lesions[C]		
EASO	IR	DPIE	Ce	Cb	Mb	Ki	He	Li	Pa	Mu	Ki	He	Mu
1	SC	5	0	+	0	+	+	++	0	0	0	0	0
2	SC	5	0	0	0	+	0	++	0	+	0	0	0
3	SC	5	0	0	0	+	0	+	0	0	0	0	0
4	SC	5	0	0	+	+	0	++	0	0	0	0	0
5[D]	SC	8	+	++	0	+	+++	++	+	+++	P	N,M	N,M
6[D]	SC	9	+	+	0	++	+++	++	0	++	P	N	N,M
7	SC	14	+	++	+	++	++	+	0	++	P	A,N	0
8	SC	14	0	0	0	+	+	+++	0	0	P	A	0
9	SC	14	+	++	0	++	++	+	++	+++	0	A	N
10	O	14	0	0	0	+	+	0	0	0	0	A	0

[A] Ce = cerebrum; Cb = cerebellum; Mb = midbrain; Ki = kidney; He = heart; Li = liver; Pa = pancreas; Mu = skeletal muscle; O = oral.
[B] 0 = No lesion observed; + = mild lesion(s); ++ = moderate lesion(s); +++ = severe lesion(s).
[C] P = phlebitis; N = necrosis; M = mineralization; A = arteritis.
[D] EASOs exhibiting WNV-related morbidity.

DISCUSSION

While there has been reference to numerous WNV-induced deaths in free-ranging and captive raptors (6,7), previous experimental infection studies in raptors have failed to elicit WNV-attributed morbidity (9,13). In one survey at a captive owl facility, there was no mortality among naturally infected EASOs, although a considerable fraction was WNV seropositive, indicating exposure and survival (7). In this study, 100% (9/9) of subcutaneously inoculated EASOs became viremic; 40% (2/5) of the initial group became clinically ill and maintained a detectable viremia for at least 7 days before death. Although previous reports of WNV-related deaths in other North American owl species have shown morbidity and mortality (6), existing reports of WNV-attributed death in EASOs are rare (7).

Morbid EASOs lost considerable weight within the several days before death and experienced relatively acute onset of severe clinical signs, evident ~12–18 hr before death. One EASO died, the second was euthanatized, and the latter followed a similar clinical course and was presumably near death when euthanatized. In a study of naturally infected zoo birds that included six raptors, 9 of 10 birds died or were euthanatized within 1–3 days after the onset of clinical signs, which included rapid weight loss, depression, and various neurologic signs (4). Another study reported that 19 of 25 (76%) of a group of great horned owls naturally infected with WNV had cachexia, and the authors suggested that many free-ranging owls might die of starvation rather than from direct effects of WNV-associated illness (17). Owls in our study were provided with abundant fresh food in close proximity to where they perched, but WNV-infected owls either failed to consume readily available food or lost weight despite food consumption. It was difficult to document food consumption by each individual in cages with an infected and noninfected cagemate because levels from all cages varied to some extent from day to day. However, overall food consumption remained relatively constant from preinfection levels until death in the cages with morbid birds and was comparable to cages with two healthy owls.



Figs. 2–7. Fig. 2. EASO 5. Photomicrograph of myocardium of a subcutaneously inoculated owl with marked myocardial necrosis. The animal died 8 DPI. H&E. Bar = 50 μm. Fig. 3. EASO 5. Photomicrograph of myocardium of a subcutaneously inoculated owl with marked cardiomyocyte mineralization. Von Kossa stain. Bar = 200 μm. Fig. 4. EASO 5. Photomicrograph of pectoral muscle of a subcutaneously inoculated owl with marked fiber necrosis. H&E. Bar = 100 μm. Fig. 5. EASO 5. Photomicrograph of pectoral muscle of a subcutaneously inoculated owl with marked fiber mineralization. Von Kossa stain. Bar = 100 μm. Fig. 6. EASO 9. Photomicrograph of myocardium (interventricular septum) of a subcutaneously inoculated owl with marked segmental necrotizing arteritis. The animal was clinically healthy until it was euthanatized 14 DPI. H&E. Bar = 50 μm. Fig. 7. EASO 7. Photomicrograph of cerebellar cortex of a subcutaneously inoculated owl with glial nodules in the molecular layer. The animal was clinically healthy until it was euthanatized 14 DPI. H&E. Bar = 100 μm.

Similar to previous attempts to infect American Kestrels (*Falco sparverius*) and Red-tailed Hawks (*Buteo jamaicensis*) with WNV by oral exposure, EASOs do not seem highly susceptible to oral infection. However, this study and others demonstrate the potential for WNV infection via the oral route for free-ranging and captive raptors (9,13) and the potential for illness following such an infection remains. The significance of oral WNV infection in free-ranging raptors remains unknown.

Viremia levels in morbid EASOs were relatively high compared with non–clinically ill birds in this study, as well as to experimentally

infected raptor species in other studies (9,13). However, even apparently healthy EASOs reached levels of viremia equal to or greater than those considered infectious to mosquitoes (15) and therefore likely serve as reservoir hosts in the WNV transmission cycle. The morbid EASOs maintained this level of viremia continuously for 5–6 days before death despite initiating an antibody response. However, owls that survived infection had at least a fourfold greater antibody titer on 7 DPI than morbid owls. The correlation between antibody levels and survival from WNV infection is unclear, and further investigation into humoral and cellular immune response to WNV infection in birds is warranted.

Although the second group of subcutaneously inoculated owls was euthanatized on 5 DPI, viremia profiles of these EASOs were consistent with those of the healthy birds from the initial group. The time between inoculations of these groups was 3 mo; it is unclear whether age difference might have affected the severity of infection and clinical outcome in these hatch-year birds. In addition, these birds had been previously exposed orally to WNV, and although they had no evidence of WNV antibodies, some priming of the immune response was a possible factor in their subsequent response to WNV infection.

Infected cagemates orally shed WNV at relatively high levels. However, no cagemate transmission occurred among EASOs despite close contact during the acute phase of infection. In a WNV experimental infection study involving American Crows (*Corvus brachyrhynchos*), noninoculated birds became infected when sharing a room with infected crows (12). Another study suggested direct bird-bird transmission of WNV among domestic geese (*Anser anser domesticus*) (1).

Application of RT-PCR to homogenized vascular feather pulp has been suggested as a sensitive method of WNV testing in corvids (5), which prompted the testing of nonpulp feathers in this study. The time frame for WNV antigen detection by RT-PCR in these feathers appears to be limited within the course of WNV infection, although the sample size was small. The viremia levels and detection of WNV antigen in feathers might be somewhat correlated because the three feather-positive birds demonstrated the highest peak viremia levels, which occurred 2–5 days before sampling of the positive feathers. Additional studies are necessary to examine the potential use of testing feathers for ante- and postmortem diagnosis of WNV infection in birds.

Viral distribution in tissues of WNV-infected EASOs varied with time and clinical outcome. Although WNV was present in most tissues of birds euthanatized on 5 DPI, the majority of tissues from birds euthanatized on 14 DPI were negative for WNV. Conversely, histologic lesions were generally mild or absent in tissues on 5 DPI, whereas more developed lesions were observed in birds euthanatized at later DPIs. This observation suggests a relatively rapid and effective clearing of WNV from tissues in survivors, with subsequent lesion development and lack of clinical signs. However in some EASOs, lesions became severe by 8–9 DPI; the viral load in many tissues reached relatively high levels, and the EASOs succumbed to WNV infection. For the two EASOs that died from WNV infection, viral loads were highest and histologic lesions most severe in the myocardium and skeletal muscle (Tables 2, 3).

In contrast to heart and skeletal muscle tissue, relatively little or no virus was isolated from brain tissue, and brain lesions were mild or absent in EASOs (Tables 2, 3). This is consistent with previous observations in owls that brain had low levels of inflammation and immunohistochemistry (IHC) staining relative to other tissues (6), and brain tissue from 48% of clinically ill, naturally infected great horned owls tested positive by RT-PCR, with 44% of these positive by IHC (17). Kidney was the most consistent organ from which WNV antigen was detected by IHC in naturally infected Great Horned Owls (17) and was also the tissue from which WNV was most commonly isolated in our study (90%; Table 2), although most of these birds had no clinical signs, unlike in the previous study.

Grossly, tissues of apparently healthy WNV-infected EASOs were normal, consistent with studies examining naturally WNV–infected raptors (6,16). The most prominent gross and histologic lesions were in the EASOs that had clinical signs of illness and involved heart and skeletal muscle. These lesions were disseminated and severe, consistent with a life-threatening process. Histologically, myocardial and skeletal muscle necrosis and mineralization was extensive and lacked prominent leukocyte infiltration, which could suggest a direct cytolytic effect of viral infection. Acute disseminated mineralization in the myocardium was observed in several birds that died or were euthanatized during a natural WNV outbreak in New York, including a Chilean Flamingo (*Phoenicopterus chilensis*) (14). Mineralization was also observed in the cerebellum and cerebrum of a naturally infected Great Horned Owl (13).

Histopathologic findings in EASOs are comparable to those observed in other WNV-infected owls in the family Strigidae (6,13,14,17) and with raptors in the family Accipitridae (16), although cerebral lesions were either mild or absent in EASOs in this study. The observation of myocardial necrotizing arteritis observed here in EASOs is similar to that previously observed in experimentally infected American Kestrels and Red-tailed Hawks euthanatized at a similar time point after WNV infection (13).

The results of this study confirm that EASOs are susceptible to acute WNV-induced disease, can become infected *per os*, and are likely competent reservoir hosts of WNV. In addition, there are key pathologic lesions of WNV infection in EASOs, such as myocardial and skeletal muscle mineralization and severe myocarditis and myocardial arteritis, with varying clinical outcomes. Although actual numbers of free-ranging EASOs and other raptors affected by WNV are not known, it is clear that WNV has the potential to gravely affect wild and captive populations. Many questions regarding WNV in raptors remain, creating the need to investigate additional routes of infection, species differences, long-term survival and breeding performance of infected birds, and characterization of protective immunity of infected survivors.

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