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Experimental Infection of Eastern Gray Squirrels (*Sciurus carolinensis*) with West Nile Virus

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Abstract. Eastern gray squirrels (*Sciurus carolinensis*) have shown high West Nile virus (WNV) seroprevalence, and WNV infection has been suggested as a cause of morbidity and mortality in this species. We experimentally infected nine eastern gray squirrels with WNV to determine the clinical effects of infection and to assess their potential role as amplifying hosts. We observed no morbidity or mortality attributable to WNV infection, but lesions were apparent in several organs. We detected mean viremias of $10^{5.1}$ and $10^{4.8}$ plaque-forming units (PFU)/mL on days 3 and 4 post-infection (DPI) and estimated that ~2.1% of *Culex pipiens* feeding on squirrels during 1–5 DPI would become infectious. Thus, *S. carolinensis* are unlikely to be important amplifying hosts and may instead dampen the intensity of transmission in most host communities. The low viremias and lack of mortality observed in *S. carolinensis* suggest that they may be useful as sentinels of spillover from the enzootic amplification cycle.

INTRODUCTION

West Nile virus (WNV) is primarily maintained in an enzootic cycle involving birds and ornithophilic mosquitoes in which mammals are considered incidental hosts.[1] Disease caused by WNV is most commonly reported in horses, humans, and some bird species (e.g., corvids), but WNV exposure has been detected in a broad range of domestic and wild mammal species.[2–6] Most experimental infections of mammals have demonstrated viremia titers that are too low to lead to a large fraction of mosquitoes that can transmit WNV, and therefore, mammals are generally considered incompetent WNV hosts.[7–11]

Recent studies have found higher viremia titers ($> 10^5$ plaque-forming units [PFU]/mL) in experimental infections of eastern cottontail rabbits (*Sylvilagus floridanus*),[12] eastern chipmunks (*Tamias striatus*),[13] and golden hamsters (*Mesocricetus auratus*).[14] Viremias greater than ~10^5 PFU/mL are necessary to result in transmitting mosquitoes, and the percent transmitting appears to increase approximately linearly from 0% at $10^{4.6}$ PFU/mL to 50% at $10^{8.3}$ PFU/mL[1], with variability between and within *Culex pipiens* populations. Viremia titers in the most infectious species, eastern chipmunks, would result in an average of 7.2% of *Cx. pipiens* mosquitoes feeding 1–5 days post infection (DPI) becoming infectious (i.e., transmitting).[1] Although chipmunks are still a relatively poorly competent host compared with most birds except galliformes and columbiformes,[1] the possibility that some peridomestic wild mammals could be amplifying hosts for WNV is an important consideration for public health. This has raised the question of the reservoir status of species living in close proximity to humans in endemic areas.

Eastern gray squirrels (*Sciurus carolinensis*) are common throughout the eastern United States, have been introduced in certain areas of the western states, and can reach high densities in urban and suburban areas.[15] Eastern gray squirrels are also frequently exposed to WNV and WNV infection has been reported as a cause of morbidity and mortality in *S. carolinensis* and fox squirrels (*Sciurus niger*).[3,16–18] The objectives of this study were to determine the clinical effects (including morbidity and mortality) of WNV infection in the laboratory and to assess the potential role of *S. carolinensis* as a WNV amplifying host.

METHODS

Animal collection and holding. Eleven wild eastern gray squirrels were captured using baited Tomahawk live traps (48.3 × 15.2 × 15.2 cm; Tomahawk Live Trap Company, Tomahawk, WI) near Albany, New York. Upon capture, animals were chemically restrained and tagged, weighed, given a cursory health evaluation, dusted for ectoparasites, and a 0.1 mL blood sample was taken from the femoral vein. Captured animals were placed into two age categories differentiating the young of the year from older individuals. Each animal was housed individually in an unfiltered stainless steel rack cage in a biosafety level 3 facility at the Griffin Laboratories, Wadsworth Center, New York State Department of Health. Animals were fed a diet of mixed nuts, fruits, vegetables, high digestibility dry dog food, and rodent chow; water was provided *ad libitum*. Housing and handling protocols were approved by Wadsworth Center's Institutional Animal Care and Use Committee (Protocol Number 05-404).

Experimental protocol. All animals were placed in quarantine for 14 days and verified to be in good body condition and seronegative for WNV, St. Louis encephalitis virus, and Powassan virus. The experimental group consisted of 9 animals and was balanced by sex and age (4 females and 5 males, 5 juveniles and 4 adults). The 2 remaining animals (a juvenile female and an adult male) were used as uninfected controls. At the end of the 14-day quarantine period, animals in the experimental group were needle-inoculated subcutaneously with 10^5 PFU of WNV strain 03-1956 (isolated from an American crow *Corvus brachyrhynchos*, in New York in 2003). This dose is similar to the dose of virus injected by *Cx. pipiens* and *Cx. tarsalis*, and gave statistically indistinguishable viremia profiles in chickens to infection by bite from a

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single mosquito.[19,20] Uninfected controls were injected with sterile phosphate buffered saline solution.

All animals were bled daily for 7 DPI, and were observed twice daily for clinical signs of infection until the end of the experiment (14 DPI). Blood samples (0.1 mL) were obtained from the femoral vein on alternating limbs and dispensed into tubes containing 0.9 mL BA-1 medium (M199 medium with Hank's salts, 1% bovine albumin, TRIS base (tris [hydroxymethyl] aminomethane), sodium bicarbonate, 2% fetal bovine serum, 100 units/mL of penicillin, 100 mg/mL of streptomycin, 1 mg/mL of Fungizone). The diluted blood samples were immediately stored at −80°C before viremia was measured by plaque assay on African green monkey kidney (Vero) cells.[21] Urine and feces were collected opportunistically. All animals were euthanized and necropzied on 14 DPI. Tissue samples from the brain, heart, kidney, liver, muscle, skin, and spleen were collected, fixed with 10% buffered formalin, processed for histopathologic examination, and stained with hematoxylin and eosine. An additional set of tissue samples was processed immediately for the presence of WNV RNA using RNeasy Extraction Kit (Qiagen, Valencia, CA).

Laboratory analyses. Blood samples were tested for WNV antibodies by plaque-reduction neutralization assay (PRNT)[22,23] at a 1:10 dilution and using 90% neutralization cutoff to confirm infection. The urine samples were concentrated prior to RNA extraction using Centricon centrifugal filters (YM-30, Millipore, Billerica, MA). Viral RNA was extracted using QIAmp Viral RNA Mini Kit and amplified using the Qiagen One-Step RT-PCR system (Qiagen, Valencia, CA).

Statistical analyses. We estimated the WNV host competence index of squirrels over the viremic periods, approximating the percentage of mosquitoes feeding on infected squirrels that would become infectious. Komar and others[24] originally developed this formula and Kilpatrick and others[1] modified it to include viremia-transmission data from vector competence studies from four populations of *Cx. pipiens*. We calculated 95% confidence intervals (CI) for the index that incorporated variability between squirrels in daily viremia, and variability and error in the viremia-mosquito infectiousness relationship. We generated 10,000 random draws from daily viremia distributions with mean and variance estimated from the data, and used these and the viremia-infectiousness relationship fit to data from four *Cx. pipiens* populations (fraction of *Cx. pipiens* transmitting = 0.1349 Log_{10} viremia = 0.6235; N = 13; R^2 = 66.3%; P = 0.001; standard error [SE] of regression = 0.1299) to generate 10,000 predicted values, which incorporate the error of the regression of infectiousness. We took the upper and lower 2.5% of these estimates as the 95% confidence limits.

RESULTS

All nine infected animals and two controls survived until 14 DPI. Two experimentally infected animals developed a mild, self-limiting suppurative conjunctivitis on 4 and 5 DPI, but no other clinical signs were observed during the study period. Most necropsies were unremarkable and only a mild nephritis was observed in three infected animals. No additional gross findings were apparent, and all animals were in good body condition at the time of necropsy.

We detected WNV viremia in all experimentally infected animals (Table 1). Viremia titers were first detected at 1 DPI and remained detectable until 5 DPI. Average viremia was highest ($10^{5.1\pm0.4}$ PFU/mL) on 3 DPI, and the highest individual titers ($10^{5.5}$ and $10^{5.6}$ PFU/mL) were detected on 3 and 4 DPI (Table 1). Viremia titers did not differ between age classes or sexes, but sample sizes were relatively small (4–5 per group) (Table 1). Eastern gray squirrels had an average host competence index of 0.106 (95% CI, 0–0.66), indicating that on average 2.1% (95% CI, 0–13%) of *Cx. pipiens* feeding on Eastern gray squirrels during the 5 DPI would become infectious.

We observed microscopic lesions in the brain, kidney, spleen, and liver. All infected animals presented pathologic changes in the brain, including lymphoid perivascular cuffs (9/9 infected animals), gliosis in the cortex (5/9) and hippocampus (3/9), and lymphocytic meningitis (3/9). No pathologic changes in the brain were observed in the uninfected controls. A renal lesion characterized by moderate lymphocytic infiltrates was found in both infected (3/9) animals and uninfected (1/2) controls. In one of the infected animals a subacute lymphocytic and tubulo-interstitial nephritis with mild loss of tubules was apparent. In two infected animals and one control, we identified a lymphocytic infiltrate in the kidney. We observed mild lymphoid depletion in the spleen of three infected animals and one control. Minimal lymphocytic cholangitis was present in three infected animals and one control, and we observed focal inflammation in the liver of one infected and one control.

We found WNV RNA in the brain and spleen of all nine animals and in the skin over the site of injection of 8 of 9 of the infected animals (Table 2). Viral RNA was also present in the kidney (7/9 animals), striated muscle (7/9), heart (5/9), and liver (4/9) (Table 2). No viral RNA was found in any tissue in the uninfected controls. We detected WNV RNA in urine from 1 to 12 DPI and in feces from 4 to 6 DPI; shedding in feces and/or urine was found intermittently in all animals in the experimental group (Table 2) and none of the controls.

DISCUSSION

The WNV infection in *S. carolinensis* produced a moderate viremia of short duration, in a pattern resembling that found

TABLE 1

Sex, age, and West Nile virus viremia titers (Log_{10} PFU/mL) in experimentally infected eastern gray squirrels (limit of detection was 10^2 PFU/mL)

		Days post infection				
Sex*	Age†	1	2	3	4	5
M	J	< 2.0	3.7	5.3	4.7	3.3
M	J	< 2.0	4.6	5.5	4.3	4.2
M	A	< 2.0	3.4	5.1	4.9	4.5
M	A	< 2.0	3.3	4.3	4.9	4.2
M	A	< 2.0	3.4	5.1	5.6	4.4
F	J	< 2.0	3.9	5.2	4.5	3.3
F	J	< 2.0	3.5	4.7	4.1	3.3
F	J	< 2.0	3.3	5.1	5.0	2.4
F	A	2.0	4.0	5.2	5.5	4.2
Mean (± SD)			3.7 ± 0.4	5.1 ± 0.4	4.8 ± 0.5	3.8 ± 0.7

* M = males: 4.4 ± 0.72 PFU/mL; F = females: 4.2 ± 0.88 PFU/mL (repeated-measures analysis of variance [ANOVA]: $F_{1,7}$ = 0.163, P = 0.698).

† J = juveniles: 4.24 ± 0.84 PFU/mL; A = adults: 4.5 ± 0.73 PFU/mL; (repeated-measures ANOVA: $F_{1,7}$ = 1.244, P = 0.302).

TABLE 2

Viral titers (PFU equivalent) isolated in tissue, urine, and feces in *S. carolinensis* experimentally infected with WNV (limit of detection was 0.09 PFU/mL)

Sample (no. tested)	Mean ± SD Log_{10}PFU (equivalent)/g
Brain (11)	3.09 ± 0.68
Heart (11)	0.44 ± 0.35
Kidney (11)	1.09 ± 1.39
Liver (11)	0.54 ± 0.42
Muscle (11)	0.45 ± 0.711
Skin (11)	1.83 ± 0.69
Spleen (11)	2.83 ± 0.73
Urine (45)*	2.66 ± 0.95
Feces (15)*	2.24 ± 1.52

* Log_{10} PFU (equivalent)/mL.

in *S. niger*.[11] In some experimentally infected species significantly higher viremias have been detected in younger animals,[12] but in this study viremias in young of the year *S. carolinensis* were not significantly different from those of adults. Although peak viremia titers observed in this study ($10^{5.6}$ PFU/mL) have been shown to be sufficient to *infect* some mosquito species,[12,25,26] these viremias may be too low to result in a significant fraction of biting vectors becoming *infectious*, i.e., able to transmit virus. This highlights the need to consider not the infection of mosquitoes, but the fraction that can transmit virus.

Previous studies have found relatively high WNV seroprevalence in eastern gray squirrels living in WNV endemic areas,[3,6,27] and other studies have shown that a key WNV vector, *Cx. pipiens*, feeds on squirrels.[28,29] Given their abundance in close proximity to human populations[15] and their low viremia relative to the bird species with which they are most frequently associated, eastern gray squirrels may dampen the intensity of WNV transmission by feeding mosquitoes that would otherwise feed on those more competent hosts. Although this species may be fed on by some *Aedes* mosquitoes, (e.g., *Ae. albopictus* or *Ae. trivitattus* which show similar susceptibility to *Cx. pipiens*[26]) the extremely low prevalence observed in *Ae. trivittatus* in Connecticut (2 of 3,306 pools of 72,132 mosquitoes[30]; minimum infection rate [MIR] = 0.00002) and New York suggests that infection of this species by squirrels or other hosts is rare, and this species appears to be a relatively minor vector of WNV to humans in New York.[31]

Manifestations of disease caused by WNV infection of tree squirrels include neurologic signs such as ataxia, tremors, circling, lethargy, and chewing at the feet.[16–18] In this study, as in experimental infections of fox squirrels and other mammal species,[7,9–11] we observed no clinical signs or abnormal behaviors in the infected animals at any time during our experimental period. Although ocular signs are a feature of some human WNV cases[32] and may be a feature of WNV infection in dogs,[33] we cannot definitively attribute the mild conjunctivitis we observed in two infected animals to WNV infection.

As with other WNV infected species, necropsies were mostly unremarkable.[7,11] Natural WNV infection leading to severe neurologic signs and death in *S. carolinensis* and *S. niger* have also resulted in no gross pathologic changes.[16,17] The only macroscopic change we observed was mild to moderate renal congestion in 3 infected animals, which were later shown also to have microscopic kidney lesions. Nephritis was the only consistently observed lesion in naturally infected fox squirrels in Michigan.[17] However, kidney lesions in infected tree squirrels in Illinois[16] were considered independent of WNV infection and in experimentally infected fox squirrels, renal lesions were present in both infected animals and uninfected controls.[11] No pathologic changes were observed in other organs regardless of the presence or severity of microscopic lesions. Microscopic lesions in the central nervous system were indicative of viral infection and consistent with previous findings of WNV encephalitis in humans and other mammals, including tree squirrels.[11,16,17,34–36] Although cardiac lesions are often found in WNV infected mammals,[16,17,33,37] none of the squirrels in this study showed pathological changes in the heart.

Our observations indicate that animals in the experimental group were not significantly affected by WNV infection, even those individuals in which we found severe microscopic lesions. It is possible that WNV infection could aggravate previously existing conditions and cause morbidity and mortality in weak or otherwise ill animals, but that healthy tree squirrels are able to survive infection. Future studies are needed to ascertain whether WNV infection could affect survival in grey squirrels by other means, such as increasing susceptibility to predation or by limiting the capacity for foraging.

The WNV RNA persisted in tissues until the end of this study (14 DPI) and was recovered intermittently in feces (up to 6 DPI, although sampling was incomplete thereafter) and urine (up to 12 DPI). Although the kidney was found to be the best organ for reverse transcription–polymerase chain reaction (RT-PCR) testing in naturally infected tree squirrels in California,[18] we did not detect WNV RNA in the kidneys of 2 out of 9 infected animals and amplified higher levels of RNA from brain and spleen samples. Previous studies have demonstrated WNV transmission to vertebrates by ingestion of infected prey.[7,38] The persistence of viral RNA in tissues raises the possibility that ingestion of WNV infected gray squirrels could be an alternative source of infection for predator species. Although we found no evidence of lateral transmission, the squirrels were caged individually and therefore further research is needed to ascertain whether the persistent shedding of viral RNA in urine and feces could result in viral transmission.[39]

In summary, our results indicate that WNV infection is not likely to be an important cause of direct mortality for *S. carolinensis* and squirrels are more likely, in some situations, to dampen WNV transmission than to amplify it. These results, in combination with previous studies that have shown that *S. carolinensis* is frequently exposed to WNV,[3,6,27] suggest that they make an ideal sentinel for WNV transmission to mammals.

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