

## Susceptibility of Fox Squirrels (*Sciurus niger*) to West Nile Virus by Oral Exposure

Sonthaya Tiawsirisup,<sup>1</sup> Bradley J. Blitvich,<sup>2</sup> Bradley J. Tucker,<sup>3</sup> Patrick G. Halbur,<sup>4</sup>  
Lyric C. Bartholomay,<sup>3</sup> Wayne A. Rowley,<sup>3</sup> and Kenneth B. Platt<sup>2</sup>

### Abstract

Fox squirrels (*Sciurus niger*) (five of eight) were infected with West Nile virus (WNV) when challenged by the oral route with  $10^{2.3}$  or  $10^{3.4}$  plaque forming units (PFU). The mean maximum serum WNV titer of infected fox squirrels was  $10^{5.1}$  PFU/mL and ranged from  $10^{4.6}$  to  $10^{5.6}$  PFU/mL. These levels of viremia are infectious for several mosquito vectors of WNV. This virus was also isolated from swabs of the oral and rectal cavities, and urine swabs between day 5 and 9 postexposure (p.e.) in amounts as high as  $10^{2.0}$ ,  $10^{2.8}$ , and  $10^2$  PFU, respectively. WNV RNA was detected in salivary gland and/or kidney tissue of three squirrels between day 65 and 72 p.e. in the presence of WNV neutralizing antibody, suggesting that long-term persistent infection occurs in fox squirrels. These observations justify further studies to determine if nonarthropod transmission and long-term persistent infection occur naturally in fox squirrels and contribute to trans-seasonal maintenance of WNV.

**Key Words:** Fox squirrels (*Sciurus niger*)—Oral exposure—West Nile virus.

**F**OX SQUIRRELS (*SCIURUS NIGER*) can develop West Nile virus (WNV) serum titers sufficient to infect several species of mosquitoes that subsequently have the potential to transmit the virus to birds, humans, and other vertebrates (Root et al. 2006, Padgett et al. 2007, Platt et al. 2008). Thus, fox squirrels could have a role in the epidemiology of WNV, especially in peridomestic settings. Observations made by Root et al. (2005) in areas of high WNV activity revealed that fox squirrels are commonly exposed to WNV. For example 49% of 53 fox squirrels representing six different locations were seropositive for WNV. This observation is consistent with the fact that fox squirrels share common habitats with birds and are exposed to both *Aedes* and *Culex* WNV vectors. Our previous study (Platt et al. 2008) also suggested that fox squirrels can be persistently infected with WNV, as the virus was recovered on days 17 and 22 post exposure (p.e.) from urine and oral swabs of two different squirrels with WNV-neutralizing antibody. WNV RNA was also detected in kidney tissue col-

lected from a third squirrel on day 29 p.e. Persistent infection (PI) with WNV has been demonstrated in golden hamsters (*Mesocricetus auratus*) and Rhesus macaques (*Macaca mulatta*). Tesh et al. (2005) demonstrated WNV in hamster urine for up to 247 days p.e. Pogodina et al. (1983) isolated WNV from organs of rhesus monkeys, including the kidney for up to 161 days p.e. As such, it is conceivable that similar long-term PI might occur in fox squirrels. If so, long-term PI of squirrels could be a mechanism for trans-seasonal WNV maintenance if periodic WNV shedding occurred in amounts sufficient to infect WNV-antibody-free squirrels by close contact, including the oral route. While fox squirrels are not considered gregarious mammals, their normal behavior would facilitate nonarthropod transmission of shed virus. For example, it is not uncommon for fox squirrels to share the same tree-hole cavity during winter months. There is also extensive interaction among males during the breeding season which in northern temperate latitudes generally occurs between the

<sup>1</sup>Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand.

<sup>2</sup>Department of Veterinary Microbiology and Preventive Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

<sup>3</sup>Department of Entomology, College of Agriculture and Life Sciences, Iowa State University, Ames, Iowa.

<sup>4</sup>Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, Iowa.

middle of December through June with peak activity in December/January and June. Females generally produce two litters of two to five individuals a year with the majority of births occurring in early spring and mid-summer (Flyger and Gates 1982). Thus, the purpose of this study was to establish whether fox squirrels are susceptible to WNV infection per os.

Two separate experiments utilizing five squirrels each were conducted to determine the infection rates, shedding patterns, and viremia profiles after oral exposure to WNV. Fox squirrels were live-trapped in Story County, IA, in the spring of 2007 and housed in Biosafety Level 3 animal facilities at Iowa State University (ISU). Squirrels were chemically restrained with ketamine:acepromazine given intramuscularly, initially at 0.44:0.44 mg/kg. This dose was subsequently adjusted for individual squirrels to provide restraint without abolishing the pharyngeal reflex. Virus inoculum was WNV strain IA02-crow (Erickson et al. 2006) in 0.5 mL of CO<sub>2</sub>-independent medium (Invitrogen, Carlsbad, CA) containing 1% fetal bovine serum. The inoculum was delivered slowly to permit swallowing. Five squirrels were challenged with 10<sup>2.3</sup> plaque forming units (PFU) of WNV, and the other five were challenged with 10<sup>3.4</sup> PFU of WNV. These virus doses were chosen because these amounts of WNV are frequently recovered from swabs of the oral cavity, rectum, and urine swabs of acutely infected squirrels (Root et al. 2006, Platt et al. 2008). Blood and swabs of the oral cavity, rectum, and urine, when possible, were collected as previously described (Platt et al. 2008) from all squirrels before inoculation and on alternate days beginning on day 1 or 2 p.e., continuing through day 9 p.e., and intermittently thereafter including the day of death. All samples were maintained at 4°C after collection until stored at -70°C. Swab and serum samples were tested for WNV by virus isolation in African Green Monkey Kidney (Vero) cells. Virus titers were measured by plaque assay, and serum antibody titers to WNV were determined by the plaque reduction neutralization test 90 (PRNT<sub>90</sub>) following standard protocols (Platt et al. 2008).

Tissues representing brain, spinal cord, salivary glands, lung, heart, liver, kidney, adrenal glands, spleen, pancreas, small intestine, and large intestine were collected from all eight squirrels at necropsy, processed for immunohistochemistry and histopathological examination using standardized

protocols at the Veterinary Diagnostic Laboratory (VDL) at ISU, and evaluated blindly by a veterinary pathologist. Virus isolation attempts were made on all tissues except intestines, from WNV-infected squirrels with the exception of squirrel 4 that died on day 17 p.e. Fresh salivary gland and kidney tissues from these squirrels were also assayed for WNV RNA, because salivary gland and kidney tissue are likely sources of potential virus shedding. These tissues were stored at -70°C in RNAlater (Ambion, Austin, TX) until processed for total RNA extraction using TRIzol LS Reagent (Invitrogen) following the manufacturer's protocol. Real-time RT-PCR was conducted by the Veterinary Diagnostic Laboratory on these tissues using a standardized protocol based on the method described by Lanciotti et al. (2000). All protocols were performed in accordance with the ISU Institutional Animal Care and Use Committee.

The response of fox squirrels to oral exposure to WNV is summarized in Table 1. Briefly, five squirrels developed WNV viremias and three failed to become viremic. Two squirrels died without a detectable viremia on day 4 p.e. during sampling and were subsequently excluded from the study. The mean maximum serum WNV titer for the five viremic squirrels was 10<sup>5.1</sup> PFU/mL and ranged from 10<sup>4.6</sup> to 10<sup>5.6</sup> PFU/mL. WNV was recovered between day 5 and 9 p.e. from swabs of the oral cavity, and rectum of four squirrels, and from urine swabs of two of these four squirrels. The maximum amount of WNV recovered from these swabs was 10<sup>2.0</sup>, 10<sup>2.8</sup>, and 10<sup>2.3</sup> PFU, respectively. This period of shedding was consistent with the shedding pattern of fox squirrels infected with WNV by needle or mosquito bite observed earlier (Platt et al. 2008), except that the onset of shedding in orally infected squirrels occurred approximately 2 days later. WNV RNA was detected by real-time RT-PCR in tissues of the kidney (two squirrels) and salivary gland (three squirrels) between day 65 and 72 p.e. Histological lesions or WNV antigen were not detected in any squirrel except squirrel 4, which died on day 17 p.e. (Table 1). WNV was not isolated from internal organs of any squirrel.

Fox squirrels are susceptible to oral infection by amounts of WNV that are commonly recovered from the oral cavity, rectum, and urine of squirrels with active infections. More importantly, the WNV serum titers observed in orally infected squirrels (Table 1) are sufficient to infect several competent

TABLE 1. RESPONSE OF FOX SQUIRRELS (*SCIURUS NIGER*) TO PER OS EXPOSURE TO WNV

Squirrel no.	WNV challenge dose <sup>a</sup>	Day of death	PRNT <sub>90</sub> titer on death day	Detection of WNV RNA		Serum WNV titer by day postexposure							
				Kidney	Salivary gland	1	2	3	4	5	6	7	9
1	3.4	78	0	No	No	-	0	-	0	-	0	-	0
2	3.4	72	160	Yes	Yes	-	-	-	5.1	-	3.0	-	0
3	3.4	65	320	Yes	Yes	0	-	0	-	4.9	-	3.2	0
4 <sup>b</sup>	3.4	17	<10	-	-	-	0	-	0	-	5.6	-	-
5	3.4	58	160	No	No	0	-	5.1	-	2.8	-	0	0
6	2.3	55	0	No	No	0	-	0	-	0	-	0	0
7	2.3	70	160	No	Yes	0	-	0	-	3.4	-	4.6	0
8	2.3	69	0	No	No	0	-	0	-	0	-	0	0

<sup>a</sup>Log<sub>10</sub> PFU/mL delivered in 0.5 mL of CO<sub>2</sub>-independent cell culture medium.

<sup>b</sup>Histological findings were mild multifocal gliosis and satellitosis in the brain and spinal cord, moderate multifocal lymphoplasmacytic and histiocytic interstitial nephritis, and mild multifocal lymphoplasmacytic hepatitis.

PRNT<sub>90</sub>, plaque reduction neutralization titer<sub>90</sub> expressed as the reciprocal of the final serum dilution that reduced plaque counts by 90%; -, not done.

species of *Culex* and *Aedes* mosquitoes (Sardelis et al. 2001, Turell et al. 2001, Goddard et al. 2002, Tiawsirisup et al. 2008). It is important to note that the levels of WNV viremia that occurred in these orally infected squirrels may have been greater than observed because of sampling constraints. Although the detection of WNV RNA in kidney and salivary gland tissue at 65 to 72 days p.e. in the absence of infectious virus is not proof of PI, it is suggestive and justifies further studies to determine if long-term PI with periodic shedding occurs in fox squirrels.

### Acknowledgments

The research reported in this article was supported by the Centers for Disease Control and Prevention Cooperative Agreement Number 1 R01 C1000267-01 for Applied Research in Emerging Infections: Investigations of West Nile virus. This is a journal paper of the Iowa Agriculture and Home Economic Experiment Station, Ames, IA (Project 5033), supported in part by the Hatch Act and State of Iowa funds. We thank Flor Fabiosa for providing technical assistance.

### Disclosure Statement

The contents are the sole responsibility of the authors and do not necessarily represent the official views of the Centers for Disease Control and Prevention. No competing financial interests exist.

### References

Erickson, SM, Platt, KB, Tucker, BJ, et al. The potential of *Aedes triseriatus* (Diptera: Culicidae) as an enzootic vector of West Nile virus. *J Med Entomol* 2006; 43:966–970.

Flyger, V, Gates, JE. Fox and Gray Squirrels. In: *Wild Mammals of North America. Biology, Management, Economics*. Chapman, JA, Feldhamer, GA, (eds). Baltimore, MD: The John Hopkins University Press, 1982:209–222.

Goddard, LB, Roth, AE, Reisen, WK, et al. Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis* 2002; 8:1385–1391.

Lanciotti, RS, Kerst, AJ, Nasci, RS, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol* 2000; 38:4066–4071.

Padgett, KA, Reisen, WK, Kahl-Purcell, N, et al. West Nile virus infection in tree squirrels (Rodentia:Sciuridae) in California, 2004–2005. *Am J Trop Med Hyg* 2007; 76:810–813.

Platt, KB, Tucker, BJ, Halbur, PG, et al. Fox squirrels (*Sciurus niger*) develop West Nile virus viremias sufficient for infecting select mosquito species. *Vector Borne Zoonot Dis* 2008; 8:225–233.

Pogodina, VV, Frolova, MP, Malenko, GV, et al. Study on West Nile virus persistence in monkeys. *Arch Virol* 1983; 75:71–86.

Root, JJ, Hall, JS, McLean, RG, et al. Serologic evidence of exposure of wild mammals to flaviviruses in the central and eastern United States. *Am J Trop Med Hyg* 2005; 622–630.

Root, JJ, Oesterle PT, Nemeth, NM, et al. Experimental infection of fox squirrels (*Sciurus niger*) with West Nile Virus. *Am J Trop Med Hyg* 2006; 75:697–701.

Sardelis, MR, Turell, MJ, Dohm, DJ, et al. Vector competence of selected North American *Culex* and *Coquillettidia* mosquitoes for West Nile virus. *Emerg Infect Dis* 2001; 7:1018–1022.

Tesh, RB, Siirin, M, Guzman, H, et al. Persistent West Nile virus infection in the goldenhamster: studies on its mechanism and possible implications of other flavivirus infections. *J Infect Dis* 2005; 192:287–295.

Tiawsirisup, S, Platt, KB, Evans, RB, et al. Vector competence of *Aedes vexans* (Diptera: Culicidae) for West Nile virus and potential as an enzootic Vector. *J Med Entomol* 2008; 45:452–457.

Turell, MJ, O'Guinn, ML, Dohm, DJ, et al. Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *J Med Entomol* 2001; 38:130–134.

Address correspondence to:

Kenneth B. Platt  
 Department of Veterinary Medicine and Preventive Medicine  
 College of Veterinary Medicine  
 Iowa State University  
 Ames, IA 50011

E-mail: kbplatt@iastate.edu

