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SHORT REPORT: FOX SQUIRREL (*SCIURUS NIGER*) ASSOCIATIONS WITH WEST NILE VIRUS

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Abstract. Tree squirrels (*Sciurus* spp.) have been recently shown to be commonly exposed to West Nile virus (WNV). Many characteristics of WNV infections in tree squirrels are unknown. To better understand WNV associations in fox squirrels (*S. niger*), we conducted mark-recapture sampling ($N = 72$) and radio telemetry to study the longitudinal seroprevalence, seroconversions, and ectoparasites of these animals during 2005–2006 in northern Colorado. Five seroconversions were documented during this study. The majority of seroconversions occurred during the late summer/fall months. However, one seroconversion was documented over the time period of February to late March 2005. Fleas (*Orchopeas howardi*) were tested for WNV RNA using real-time PCR techniques. No WNV RNA positive fleas ($N = 33$) were detected. In addition, urine samples ($N = 17$) opportunistically collected from fox squirrels were negative for WNV RNA. Results indicate that seroconversions can be observed in fox squirrels during low WNV transmission years.

Although West Nile virus (WNV) can infect a wide range of vertebrates,[1] the natural cycle of WNV involves the transmission of the virus to birds by mosquito vectors.[2] Despite the presumed lack of host competency in mammals, evidence of WNV infections has been detected in a variety of mammalian species,[3,4] indicating widespread exposure to WNV in this taxon.

Exposure of mammals to WNV may occur via several routes. For example, whereas some mosquito species are ornithophilic, others have opportunistic feeding habits, potentially bridging WNV transmission between birds and mammals.[5] Some additional scenarios include alternative vectors[6] (e.g., ticks) and prey to predator transmission among carnivores and possibly scavengers.[7,8]

The widespread exposure of mammals to WNV,[4] evidence of transmission, and a recent study indicating host competency in eastern cottontail rabbits (*Sylvilagus floridanus*)[9] argue for more extensive investigation of the roles mammals might play in the maintenance and transmission of WNV. Using mark-recapture sampling, we sampled fox squirrels ($N = 72$) for antibodies to WNV to determine their exposure to WNV from February 2005 to January 2006. Because high antibody prevalence rates have been reported for tree squirrels,[4] we also sampled fleas (*Orchopeas howardi*, $N = 26$) from fox squirrels and, with the aid of radio telemetry, their nest cavities ($N = 7$) for evidence of WNV. Our objective was to monitor wild fox squirrels longitudinally to detect seroconversions and attempt to assess alternative WNV transmission associations.

Fox squirrels are common throughout many urban and suburban communities. In Fort Collins, Colorado, fox squirrels are found in most locations with mature trees (e.g., neighborhoods or natural areas). Although some extreme examples have been observed, few fox squirrels survive more than 5 to 6 years in the wild.[10]

Sampling was conducted with live-traps on two study sites (SFC and NFC) with appropriate habitat separated by 15.6 km. The first was located in northern Fort Collins (NFC) and was a tree nursery with running water present in irrigation ditches for part of the year. The second study site was located near the Fort Collins/Loveland border (SFC). This site was a privately owned property, primarily a large lake surrounded by cottonwood trees. Attempts were made to sample fox squirrels intermittently every 2 to 4 months, logistics permitting. A total of six sampling occasions were conducted (approximately 1150 trap-nights).

Captured fox squirrels were tagged (one tag in each ear), weighed, sexed, bled (femoral vein), combed for ectoparasites (primarily during the late fall and winter months), and a small subset ($N = 7$) were radio collared (Model # M1640, Advanced Telemetry System, Isanti, MN). Recaptured fox squirrels were processed once during every sampling occasion (e.g., ≥ 1 month between processing occasions).

Fox squirrel nest locations ($N = 11$) were assessed using radio telemetry and direct observation. When nest cavities were detected, litter was removed from the nest and placed in a berlese funnel (BioQuip, Ranch Dominguez, CA) for approximately 18 hours to collect any ectoparasites. When detected, fleas were frozen at −70°C until identification and analyses.

In light of the recent findings by Tesh and others[11] indicating that WNV is shed in the urine of golden hamsters (*Mesocrietus auratus*), attempts were made to opportunistically collect urine from wild-caught fox squirrels ($N = 17$) during the last trapping session of this study (January 2006). This was accomplished by placing a tray under the trap of captured squirrels and pipetting any excreted urine into cryovials. Urine was frozen at −70°C in the laboratory prior to analyses.

Serologic analyses were conducted by established methods. These included an epitope-blocking ELISA technique[12] for serum screening and plaque reduction neutralization tests (PRNT) to confirm the presence of WNV antibodies.[13] PRNTs were performed using the live attenuated recombinant vaccines ChimeriVax-WN (for West Nile virus antigen) and ChimeriVax-SLE (for Saint Louis Encephalitis virus antigen [SLEV]; Acambis Incorporated, Cambridge, MA), which are neutralized by anti-WNV and anti-SLEV neutralizing antibodies. Serum samples were insufficient to conduct SLEV $PRNT_{90}$ assays for some samples ($N = 3$).

Prior to RNA extractions, fleas (typically $N = 1$, although small groups were occasionally pooled from the same animal

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or nest) were mechanically macerated in Bovine Albumin-1 (BA1). Following maceration, RNA isolations were attempted from fleas ($N = 33$), and urine samples ($N = 17$) using Viral RNA Mini Kits (QIAGEN, Valencia, CA). The Taqman® One-Step reverse transcriptase polymerase chain reaction (RT-PCR) system (Applied Biosystems, Foster City, CA) and the method of Lanciotti and others (2000)[14] were used to quantify WNV RNA.

During six distinct trapping sessions, we recorded 139 captures from 72 individual fox squirrels (Table 1). Fox squirrels recaptured during the same trapping session (< 1 month apart) were not sampled. Also, squirrels that escaped without being tagged are not included in the totals reported earlier. Nineteen individuals were captured from SFC, whereas 53 were captured form NFC.

Sixteen fox squirrels were documented to have WNV antibodies during their first capture. This was the case for several fox squirrels first captured during the winter, indicating that they likely were exposed to WNV during a previous transmission season. Overall, 47% (9/19) and 23% (12/52) of tested fox squirrels were antibody positive for WNV by the end of this study at SFC and NFC, respectively. In addition, one fox squirrel (SFC-5) had detectable WNV RNA in its blood during its August 2005 capture ($10^{3.7}$ pfu/ml serum RT-PCR equivalent).

A total of five WNV seroconversions were noted during the duration of this study (3 males and 2 females, 4 adults and 1 sub-adult). Three of five seroconversions occurred between two trapping sessions, with three seroconversions occurring between August and October of 2005 (SFC-5, SFC-7, NFC-1; Table 1). One of these seroconversions (SFC-7) did not yield a four-fold difference between WNV and SLEV (data not shown) $PRNT_{90}$ titers. However, a fourfold difference was noted by the next sampling period, suggesting it was recently infected with WNV when antibody was first detected. An additional seroconversion (NFC-4) occurred between August and December 2005 (this individual was not recaptured in October 2005). Another seroconversion occurred between 3 February and 31 March 2005 (SFC-4; Table 1).

West Nile virus antibodies were detected in 28% of fox squirrels tested during this project. Not surprisingly, antibody titers in WNV antibody positive fox squirrels did not wane below detectable levels during this study. In addition, two fox squirrels that were WNV antibody positive during their first capture remained antibody positive for the duration of this study (indicating, barring a second exposure, that WNV antibodies persist at least 1 year in fox squirrels; Table 1). However, the WNV PRNT titers of some individuals dropped to levels (1:20) that could not be distinguished from SLEV (< 10, data not shown) on subsequent sampling occasions.

Twenty-six fleas (*Orchopeas howardi*) were collected from 16 squirrels; an additional seven specimens were collected from 3 of 11 nest cavities surveyed. The majority of fleas were collected during the winter and spring of 2005, although opportunistic collections of fleas from fox squirrels occurred throughout the year. None of the fleas were found to be positive by RT-PCR.

Recently, Tesh and others[11] indicated that WNV-infected hamsters develop chronic viruria for up to 8 months post infection. Thus, we hypothesized that WNV antibody positive

TABLE 1

The serological profiles of fox squirrels (*Sciurus niger*) at study sites SFC and NFC, Larimer County, CO that were WNV antibody positive at the time of initial capture or subsequently seroconverted during the period of February 2005 to January 2006; data on multiple captures of 51 WNV seronegative fox squirrels are not shown

	Fox squirrels								
	Capture history*						Demographic data		
Individual	Feb-05†	Apr-05	Aug-05	Oct-05	Dec-05	Jan-06	Sex	Mass‡	Age¶
SFC-1	**A** (40)	A (1280)	A (160)	A (40)	A (40)	A (20)	F	700	A
SFC-2	**A** (80)			A (20)	A (40)	A (40)	F	750	A
SFC-3	**A** (160)						F	725	A
SFC-4	X (< 10)	**A** (40)					F	700	A
SFC-5	X		X (< 10)§	**A** (640)	A (160)	A (160)	M	750	A
SFC-6		**A** (80)					M	675	A
SFC-7			X (< 10)	**A** (20)	A (160)	A (160)	M	425	S
SFC-8			**A** (640)				F	600	A
SFC-9				**A** (40)	A (80)	A (20)	M	760	A
NFC-1	X		X (< 10)	**A** (640)	A (320)		M	775	A
NFC-2	**A** (320)						F	700	A
NFC-3		**A** (160)					F	775	A
NFC-4		X	X (< 10)		**A** (40)		F	550	A
NFC-5		**A** (160)	A (80)		A (40)	A (40)	M	750	A
NFC-6			**A** (160)	A (160)		A (80)	M	750	A
NFC-7			**A** (80)		A (40)		M	660	A
NFC-8				**A** (40)			M	660	A
NFC-9				**A** (1280)		A (320)	M	680	A
NFC-10					**A** (640)		M	680	A
NFC-11					**A** (1280)		M	620	A
NFC-12					**A** (80)	A (40)	F	600	A

* An "X" indicates a sampling occasion when a fox squirrel is captured. A bold "**A**" indicates the first sampling occasion when antibodies to West Nile virus were detected in that individual and a standard "A" indicates that the individual was still antibody positive during that sampling period. The number in parenthesis indicates the WNV antibody titer as determined by plaque reduction neutralization tests ($PRNT_{90}$).

† Month indicates the month when the majority of the trapping occurred. Occasionally months overlapped by a few days. Days were not always consecutive. Feb-05 = SFC = 2/1/05–2/4/05, NFC = 2/10/05; Apr-05 = SFC = 3/31/05–4/1/05, NFC = 4/7/05–4/8/05; Aug-05 = SFC = 8/11/05–8/12/05, NFC = 8/15/05–8/19/05; Oct-05 = SFC and NFC = 10/3/05–10/6/05; Dec-05 = SFC = 11/19/05–12/13/05, NFC = 12/1/05–12/13/05; Jan-06 = SFC and NFC = 1/23/06–1/27/06.

‡ Grams at first capture.

¶ Ages (A = adult; S = sub-adult) were determined based on mass, season, and appearance.

§ Individual was virmic during its August capture ($10^{3.7}$ pfu/ml serum RT-PCR equivalent).

wild fox squirrels might still be shedding WNV in their urine. Seventeen urine samples were opportunistically collected from fox squirrels during the last trapping session of the study (January 2006); none were found to be positive by RT-PCR, even though several ($N = 7$) of the samples tested were from known WNV-exposed squirrels. Because of the small sample size reported herein, these data are inconclusive.

During this study, a single fox squirrel was documented to have seroconverted during late winter/early spring. Furthermore, a different fox squirrel (SFC-1; Table 1) was WNV antibody positive (1:40) during February 2005, but its titer increased significantly (1:1280) by 1 April 2005, which may indicate a subsequent winter/early spring WNV exposure. For example, Root and others[15] detected a minimal antibody response in most experimentally infected fox squirrels by 10 days post infection. A second exposure may represent another potential scenario for a rapid rise in antibody titers of flaviviruses.[16] Viral recrudescence is a possibility as well. However, early season mosquito transmission cannot be ruled out. For example, some *Culex* spp. were collected in Larimer (study site locations) and Weld (adjacent County) counties in April and November of 2004; however, their numbers were significantly reduced when compared with peak months (B. Bolling, Colorado State University, unpublished data). Mosquito collection sites were within 5.9 and 11.8 km of NFC and SFC, respectively. In addition, *Culex tarsalis* have been detected as early as March in nearby Loveland (M. Doyle, Larimer County Health Department, unpublished data; approximately 8.5 km from SFC). Nonetheless, historical data from adjacent areas in Colorado suggests that engorgement of *Culex tarsalis* is low until mid to late April and drops to very low rates by October.[17]

As determined by reported human cases, 2005 was a low year of WNV activity in Colorado, with 101 cases reported as of 14 February 2006.[18] The transmission season of 2004 also yielded relatively few human cases, with 291 being reported.[19] In contrast, 2003 was a high transmission year, with 2947 human cases being reported.[20] Of interest, we were able to detect five seroconversions among 72 fox squirrels at two small study sites during a very low WNV transmission year in Colorado.

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REFERENCES

1. Centers for Disease Control and Prevention, 1999. Outbreak of West Nile-like viral encephalitis—New York, 1999. *MMWR 48:* 845–849.
2. McLean RG, Ubico SR, Bourne D, Komar N, 2002. West Nile virus in livestock and wildlife. *Curr Top Microbiol Immunol 267:* 271–308.
3. Dietrich G, Montenieri JA, Panella NA, Langevin S, Lasater SE, Klenk K, Kile JC, Komar N, 2005. Serologic evidence of West Nile virus infection in free-ranging mammals, Slidell, Louisiana, 2002. *Vector Borne Zoonotic Dis 5:* 288–292.
4. Root JJ, Hall JS, McLean RG, Marlenee NL, Beaty BJ, Gansowski J, Clark L, 2005. Serologic evidence of exposure of wild mammals to flaviviruses in the central and eastern United States. *Am J Trop Med Hyg 72:* 622–630.
5. Goddard LB, Roth AE, Reisen WK, Scott TW, 2002. Vector competence of California mosquitoes for West Nile virus. *Emerg Infect Dis 8:* 1385–1391.
6. Abbassy MM, Osman M, Marzouk AS, 1993. West Nile virus (Flaviviridae: *Flavivirus*) in experimentally infected *Argas* ticks (Acari: Argasidae). *Am J Trop Med Hyg 48:* 726–737.
7. Garmendia AE, Van Kruiningen HH, French RA, Anderson JF, Andreadis RG, Kuman A, West B, 2000. Recovery and identification of West Nile virus from a hawk in winter. *J Clin Microbiol 38:* 3110–3111.
8. Austgen LE, Bowen RA, Bunning ML, Davis BS, Mitchell CJ, Chang G-JJ, 2004. Experimental infection of cats and dogs with West Nile virus. *Emerg Infect Dis 10:* 82–86.
9. Tiawsirisup S, Platt KB, Tucker BJ, Rowley WA, 2005. Eastern cottontail rabbits (*Sylvilagus floridanus*) develop West Nile virus viremias sufficient for infecting select mosquito species. *Vector Borne Zoonotic Dis 5:* 342–350.
10. Fitzgerald JP, Meaney CA, Armstrong DM, 1994. *Mammals of Colorado*. Niwot, CO: University Press of Colorado, 467 pp.
11. Tesh RB, Siirin M, Guzman H, Travassos da Rosa APA, Wu X, Duan T, Lei H, Nunes MR, Xiao S-Y, 2005. Persistent West Nile virus infection in the golden hamster: studies on its mechanism and possible implications for other flavivirus infections. *J Infect Dis 192:* 287–295.
12. Blitvich BJ, Bowen RA, Marlenee NL, Hall RA, Bunning ML, Beaty BJ, 2003. Epitope-blocking enzyme-linked immunosorbent assays for detection of West Nile virus antibodies in domestic mammals. *J Clin Microbiol 41:* 2676–2679.
13. Beaty BJ, Calisher CH, Shope RE, 1995. Arboviruses. Lennette EH, Lennette DA, Lennette ET, eds. *Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections*. Seventh edition. Washington, DC: American Public Health Association, 89-212.
14. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, Davis BS, Roehrig JT, 2000. 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 38:* 4066–4071.
15. Root JJ, Oesterle PT, Nemeth NM, Klenk K, Gould D, McLean RG, Clark L, Hall JS. Experimental infection of fox squirrels (*Sciurus niger*) with West Nile virus. *Am J Trop Med Hyg 75:* 697–701.
16. Reisen WK, Chiles RE, Green EN, Fang Y, Mahmood F, 2003. Previous infection protects house finches from re-infection with St. Louis encephalitis virus. *J Med Entomol 40:* 300–305.
17. Benington EE, Sooter CA, Baer H, 1958. The diapause in adult female *Culex tarsalis* coquillett (Diptera: Culicidae). *Mosquito News 18:* 299–304.
18. U.S. Geological Survey, 2005. http://westnilemaps.usgs.gov/2004/co_human.html. Accessed 12/13/2005.
19. U.S. Geological Survey, 2005. http://westnilemaps.usgs.gov/2003/co_human.html. Accessed 12/13/2005.
20. U.S. Geological Survey, 2006. http://westnilemaps.usgs.gov/co_human.html. Accessed 02/14/2006.