The Journal of Raptor Research

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J. Raptor Res. 43(4):301–307

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ABSTRACT.—American Kestrels (*Falco sparverius*) nesting near Hawk Mountain Sanctuary in eastern Pennsylvania exhibited widespread West Nile virus (WNV) seroprevalence in 2004. Here we examine the dynamics of WNV in kestrels nesting near Hawk Mountain Sanctuary in 2004–06. We tested kestrel saliva for viral RNA using oral swabs analyzed by RT-PCR, and all swabs were negative for WNV. We used plaque-reduction neutralization testing (PRNT) to measure WNV antibodies in serum. In 2004, we tested 22 adult kestrels and most (95%) were positive for WNV. In 2005, we tested blood samples from six adults and seven nestlings, and WNV seroprevalence was 33% in adults and 14% in nestlings. In 2006, we sampled seven adults and eight nestlings, and 29% of the adults and no nestlings were positive. WNV was widespread in birds, mosquitoes, and humans in Pennsylvania in 2003, and subsequently has declined in these populations. The prevalence of WNV antibodies in the kestrels was compared to surveillance data for mosquitoes, dead birds, and humans in Pennsylvania. WNV antibody prevalence in kestrels was significantly correlated with prevalence of WNV infection in dead birds from the previous year (correlation coefficient 1.000; $P = 0.009$), but not with prevalence in mosquitoes or humans from the previous year or in dead birds, humans, and mosquitoes from the same year. This suggested that the antibody levels observed in the kestrel population we studied resulted from infections that occurred during the previous year. Our data indicated that WNV prevalence has declined in this population of kestrels over time.

KEY WORDS: *American Kestrel*; Falco sparverius; *Pennsylvania*; *raptor*; *seroprevalence*; *West Nile virus*.

VARIACIÓN ANUAL DE LOS ANTICUERPOS CONTRA EL VIRUS DEL OESTE DEL NILO EN *FALCO SPARVERIUS* EN EL ESTE DE PENSILVANIA

RESUMEN.—Los individuos de la especie *Falco sparverius* que anidan cerca del santuario Hawk Mountain en el este de Pensilvania exhibieron una amplia seropevalencia del Virus del Oeste del Nilo (VON) en 2004. Aquí examinamos la dinámica del VON en aves que anidaron cerca del santuario Hawk Mountain entre 2004 y 2006. Hicimos pruebas para detectar ARN viral tomando una muestra de saliva de cada individuo y analizándolas con PCR-TR. Todas estas muestras fueron negativas para el VON. Realizamos pruebas de neutralización por reducción de placas para medir los anticuerpos contra el virus en el suero. En 2004, muestreamos 22 individuos adultos de *F. sparverius* y la mayor parte de éstos (95%) fueron positivos para el VON. En 2005, muestreamos sangre de seis adultos y siete polluelos para los cuales la seroprevalencia fue de 33% y 14%, respectivamente. En 2006, muestreamos siete adultos y ocho polluelos, para los cuales la seroprevalencia fue del 29% para los adultos y 0% para los polluelos. En 2003, el VON tuvo una distribución amplia entre las aves, mosquitos y humanos en Pensilvania, pero ha disminuido desde entonces en estas poblaciones. La prevalencia de anticuerpos para VON en *F. sparverius* en Pensilvania fue comparable a la de los datos de monitoreo de mosquitos, aves muertas y humanos. La prevalencia de anticuerpos para VON en *F. sparverius* se correlacionó significativamente con la prevalencia de infecciones por VON en aves muertas del año anterior (coeficiente de correlación 1.000; $P = 0.009$), pero no se correlacionó con la prevalencia del virus del año anterior en mosquitos o en seres humanos, ni con la prevalencia en mosquitos

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del mismo año. Esto sugirió que los niveles de anticuerpos que se observaron en la población de *F. sparverius* que estudiamos fueron el resultado de infecciones que ocurrieron durante el año anterior. Nuestros datos indican que a lo largo del tiempo la prevalencia del VON ha disminuido en esta población.
[Traducción del equipo editorial]

West Nile virus (WNV) was first observed in the United States in September 1999, when a large number of dead crows (*Corvus* spp.) and other birds were observed in the New York City area. This was followed by human and equine encephalitis infections that later were identified as the same strain of WNV that had caused mortality in the birds (Lanciotti et al. 1999). WNV infection has resulted in the death of at least 100,000 crows and other birds, and some regions that previously had large bird populations have had noticeable declines in population densities (Malakoff 2002, LaDeau et al. 2007). However, the overall effect of WNV on bird populations, particularly in migratory birds, remains unclear (LaDeau et al. 2007, Saito et al. 2007). Such effects are difficult to determine, primarily because we lack good estimates of current population numbers or of the numbers of birds prior to the introduction of WNV. Crows and some raptor species may be especially susceptible to the disease (Andreson et al. 1999, Malakoff 2002, Nemeth et al. 2007a, Saito et al. 2007), and WNV infection has been implicated as the cause of increased clinical admissions and deaths in raptors observed by wildlife-rehabilitation centers in the United States (Joyner et al. 2006, Nemeth et al. 2007a, Saito et al. 2007).

American Kestrels (*Falco sparverius*) are widely distributed secondary cavity nesters that sometimes nest in human-made nest boxes. Nest-box use has facilitated the study of their reproductive biology for a number of years (Katzner et al. 2005). Consequently, population data are available for kestrels both from before and after the appearance of WNV. A reduction in kestrel numbers has been observed along the Atlantic Coast of the eastern United States (Sullivan and Wood 2005), as well as in our study area in eastern Pennsylvania (Medica et al. 2007, Farmer et al. 2008). Several possible explanations have been suggested for this decline, including habitat loss, organophosphate poisoning, increased predation by expanding populations of Cooper's Hawks (*Accipiter cooperii*; Farmer et al. 2006), loss of potential nesting holes due to declines of Northern Flickers (*Colaptes auratus*), and WNV infection (Sullivan and Wood 2005).

WNV has been monitored in dead birds and mosquitoes in Pennsylvania since 2000, and is widespread in the state, having been detected in all counties by 2003 (Helwig 2005, Pennsylvania WNV Surveillance Program 2008). Our objective was to measure WNV exposure in the American Kestrels breeding in nest boxes in the region around Hawk Mountain Sanctuary in 2004–06.

Data from the first year of this study (2004) indicated a high level of exposure of American Kestrels to WNV (Medica et al. 2007). The current study was designed to determine whether the prevalence of WNV antibodies (seroprevalence) in this population of kestrels varied over time. It has been proposed that the monitoring of dead raptors and live kestrels and other raptors admitted to wildlife-rehabilitation centers may serve as an indication of WNV activity in an area (Joyner et al. 2006, Nemeth et al. 2007a, 2007b, Saito et al. 2007). The seroprevalence of WNV in the kestrels in this study is compared to data for humans, mosquitoes, and dead birds to determine whether this population of breeding kestrels may be used as an indicator of WNV activity in eastern Pennsylvania.

METHODS

Study Site and Sample Population. The study site is a 1500-km^2 mainly agricultural area surrounding Hawk Mountain (eastern Pennsylvania; 40°30′N, 75°50′W) that includes 140 nest boxes designed for kestrels. Kestrels nesting in this area are partial migrants; some individuals remain in the area year-round, whereas others migrate south out of the area in winter, and still others migrate from the north into the area in winter. Sanctuary scientists and volunteers monitor activity at the nest boxes as part of an ongoing study of the reproductive biology of the American Kestrel begun in 1992 (for details see Katzner et al. 2005).

We trapped adult kestrels from 8 June–22 July in 2004, 15 June–12 July in 2005, and 6 June–22 June in 2006 using either bal-chatri traps baited with mice (*Mus musculus*), or with mist nests and an artificial owl set near the nest box. After capture, oral swabs were collected and blood samples (0.6 ml) were taken from the jugular vein and handled as described by Komar et al. (2001). All kestrels were sexed, weighed to the nearest gram, banded with a uniquely numbered U.S. Geologic Survey alumi-

num leg band, and released. Nestlings 15–23 d old were removed from nest boxes, sexed (when possible), weighed, and banded. We obtained oral swabs (below), estimated nestling age according to the degree of wing and tail feather development (Griggs and Steenhof 1993) and then returned nestlings to the nest box. In 2005 and 2006, we collected approximately 0.6 ml of blood from the largest nestling at each nest.

Oral Swabs to Test for Active Infection. WNV particles are secreted in the saliva of infected birds (Komar et al. 2003). To test for active infection in nestlings and trapped adults, we used sterile cotton-tipped swabs to swab the interior of the mouth and throat of each bird. We placed each swab in an 11-ml Rohre tube (Sarstedt, Germany) containing 3 ml of BA-1 medium and then capped it. The samples were kept on ice until the end of the day and then frozen at −20°C (2004) or −80°C (2005, 2006) until shipment to the Pennsylvania Department of Health for analysis by reverse transcriptase polymerase chain reaction (RT-PCR) to test for viral RNA. The RNA extraction and PCR protocols were those used by the Department of Health, Bureau of Laboratories, Virology and Immunology Section, West Nile Virus Surveillance Program of Pennsylvania. The methods used to measure WNV RNA were modified from previously published studies (Lanciotti et al. 2000, Shi et al. 2001, Komar et al. 2002). The primers and probes listed in Table 1 were used in detecting the presence of WNV genomic material in samples. All probes were dual-labeled using 5′-FAM (reporter dye) and 3′-TAMRA (quencher dye). All primers and probes were obtained from Eurogentec North America, Inc. (San Diego, California, U.S.A.). All specimens were first tested using the WNV3 series of primers and probes. After the RT-PCR was completed samples were identified as follows: positive, CT (threshold cycle) value ≤ 37; negative, CT value ≥ 40; and inconclusive, $37 <$ CT value < 40.

Tentative positive and inconclusive samples were re-extracted and retested by RT-PCR using the WNV2 series of primers and probe and, if necessary, re-extracted and retested by RT-PCR using the WNV1 series of primers and probe.

Sample Prep for WNV Antibody Testing. We determined the exposure of kestrels to WNV by testing serum samples using Plaque Reduction Neutralization Tests (PRNT). This is similar to the method used in avian seroprevalence studies following the initial WNV outbreak in the New York City area (Komar et al. 2001). PRNT assays were conducted at the New York State Animal Health Diagnostic Laboratory as described previously (Medica et al. 2007).

Table 1. Primer and probe sets used in RT-PCR analysis to test for West Nile virus in oral swabs of American Kestrels, eastern Pennsylvania, 2004–06.

PROBE SET NAME	PRIMER SEQUENCE (5′ → 3′)[1]
WNV1-F	CAGACCACGCTACGGCG
WNV1-R	CTAGGGCCGCGTGGG
WNV1-P	FAM-TCTGCGGAGAGTGCAGTCTGCGAT-TAMRA
WNV2-F	TCAGCGATCTCTCCACCAAAG
WNV2-R	GGGTCAGCACGTTTGTCATTG
WNV2-P	FAM-TGCCCGACCATGGGAGAAGCTC-TAMRA
WNV3-F	GCTCCGCTGTCCCTGTGA
WNV3-R	CACTCTCCTCCTGCATGGATG
WNV3-P	FAM-TGGGTCCCTACCGGAAGAACC-ACGTT-TAMRA

[1] FAM = 6-carboxy-fluorescein, TAMRA = 6-carboxy-tetramethyl-rhodamine.

Data Analysis. We compared WNV seroprevalence in kestrels to that of mosquitoes (primarily *Culex* spp.), dead birds (primarily corvids reported dead to the state health department), and humans in the state of Pennsylvania during 2003, 2004, 2005, and 2006 (data obtained from Pennsylvania WNV Surveillance Program 2008). WNV seroprevalence in kestrels was compared to that of the surveillance populations from the same year and of the previous year. Data were not normally distributed, so we calculated the nonparametric Spearman correlation coefficient (rho) using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, U.S.A.) and set the significance level at $P \leq 0.05$.

RESULTS

Sample Population. In 2004, we trapped 22 American Kestrels at 18 of the 49 nest boxes where adults were breeding (both male and female adults were captured at two sites). In 2005, adults were trapped at six, and nestlings were tested at seven (a total of 10 sites), of the 35 boxes where kestrels bred that year. In 2006, adults were trapped at eight and young were sampled at seven of the 38 boxes where kestrels bred that year.

RT-PCR Analysis of Oral Swabs. In 2004, we tested a total of 17 adults (7 male and 10 female) for active infection using oral swabs. Oral swabs were

Table 2. Prevalence of antibodies to West Nile virus declined in American Kestrels during 2004–06 in eastern Pennsylvania. In 2004, 95% of adults tested positive (titer of 1:40 or higher). In 2005, 33% of adults and 14% of nestlings tested positive. In 2006, 29% of the adults and no nestlings were seropositive. The number of individuals with titers greater than 1:80 also declined during this study.

			NUMBER WITH TITERS OF						
YEAR	AGE	SEX	<1:40	1:40	1:80	1:160	1:320	1:≥640	POSITIVE TITER (%)
2004	Adult	M	1	1	1	3	2	0	88
		F	0	2	1	7	2	2	100
2005	Adult	M	2	2	0	0	0	0	50
		F	2	0	0	0	0	0	0
	Nestlings	M	2	0	1	0	0	0	33
		F	5	0	1	0	0	0	17
2006	Adult	M	3	1	0	0	0	0	25
		F	2	1	0	0	0	0	33
	Nestlings	M	2	0	0	0	0	0	0
		F	7	0	0	0	0	0	0

not obtained for four of the adults because the testing materials were not available at the time of capture. We also swabbed 41 nestlings (21 male and 20 female) in 2004. We obtained oral swabs from six adults (two male, four female) and 28 nestlings (14 male, 14 female) in 2005, and eight adults (five male, three female) and 48 nestlings (22 male, 25 female, 1 too young to sex) in 2006. The RT-PCR analyses of all oral swabs were negative, whereas positive controls for all assays performed as expected.

West Nile Virus Antibody Levels. WNV seroprevalence in our population was quite high (95%) during the first year of this study, and declined each year thereafter (Table 2). In 2004, WNV PRNT analysis resulted in positive antibody titers ranging from 1:40–1:≥640 for adult females and 1:40–1:320 for adult males; only one adult male tested negative for WNV antibodies. In 2005, only two adult males were positive, both with titers of 1:40, all adult females were negative, and one male and one female nestling had titers of 1:80. In 2006, one adult male and one adult female were positive with titers of 1:40, and all of the nestlings were negative. All samples were negative for St. Louis encephalitis (SLE), indicating that there was no cross-reactivity with SLE in this assay.

Data Analysis. WNV seroprevalence was not significantly correlated with the numbers of WNV-positive dead birds, mosquitoes, or humans from the same year (Table 3). Seroprevalence was positively correlated to the number of WNV-positive dead birds from the previous year, but there were no significant correlations between kestrel seroprevalence and the numbers of infected mosquitoes or humans in the previous year (Table 3).

DISCUSSION

New and reemerging infectious diseases have the potential to devastate wildlife populations, especially in situations where the animals have no innate immunity or prior exposure to the disease (Dobson and Foufopoulos 2001). When WNV entered the continental United States in 1999, resident avian populations had no immunity to the disease, as evidenced by massive die-offs of crows and some other species. WNV infection has been implicated as the cause of death in a number of raptor species (Gancz et al. 2004, Nemeth et al. 2007a, Saito et al. 2007), including American Kestrels. Eleven of 33 kestrels found dead in upstate New York tested positive for WNV (Chu et al. 2003).

For kestrels nesting near Hawk Mountain, breeding success has remained relatively constant, but the number of pairs breeding in monitored nest boxes has declined since 1999, which coincided with the introduction of WNV into New York City and surrounding areas. Data from the first year of the current study (see Medica et al. 2007) suggested that although WNV cannot be implicated as the cause of this decline, it may be a contributing factor. During the first year of this study (2004), WNV seroprevalence in kestrels was much higher than that published for other species of birds (Medica et al. 2007). Almost all (95%) of the adult kestrels tested

Table 3. Spearman correlation between West Nile virus seroprevalence in American Kestrels and the numbers of infected dead birds, mosquitoes, and humans during the same year (current) and the previous year, eastern Pennsylvania, 2004–06.

COMPARISON GROUP	YEAR	R_S	P
Dead birds	Current	−0.500	0.667
	Previous	1.000	0.009*
Mosquitoes	Current	−0.500	0.667
	Previous	0.984	0.113
Humans	Current	0.500	0.667
	Previous	0.996	0.058

* Significant correlation.

had detectable levels of antibodies, and the prevalence was much lower during subsequent years (33% in 2005 and 29% in 2006). In addition, we observed the highest antibody titers in 2004, and these were lower each subsequent year (see Table 2). This suggests that exposure of kestrels to WNV in this part of Pennsylvania has decreased over time.

Seroprevalence in adult kestrels was compared with the numbers of infected dead birds, mosquitoes, and humans in Pennsylvania for two reasons. First, comparison of kestrel seroprevalence data with surveillance data may give some indication of the cause of the decrease in seroprevalence observed between 2004 and 2006. Second, it has been suggested that raptors may be good indicators of WNV in geographic areas where crows are not commonly found (Eidson et al. 2001) and that monitoring clinic-admitted WNV-infected raptors may contribute to current surveillance methods (Nemeth et al. 2007b). Our system allows us to obtain blood samples and oral swabs from adult and nestling kestrels in known locations (near the nest boxes), and thus could also serve as a method of enhanced local WNV surveillance.

None of the adult or nestling birds in this study exhibited clinical signs of WNV infection, and all oral swabs used to test for active WNV infection were negative. WNV-infected kestrels generally shed virus for approximately 10 d (Komar et al. 2003), so the probability of trapping an infected kestrel that was actively shedding virus was relatively low in this type of study. WNV is not generally observed in birds and mosquitoes in Pennsylvania until late July (Pennsylvania WNV Surveillance Program 2008), and as we tested our birds before that date each year, this further reduced the chances of our finding an infected individual.

Kestrels may be exposed to WNV in Pennsylvania in late summer before they migrate, elsewhere during their autumn and spring migrations, or on their wintering grounds. Hull et al. (2006) found high levels of WNV antibodies in migrating and overwintering Red-tailed Hawks (*Buteo jamaicensis*), Red-shouldered Hawks (*B. lineatus*) and Cooper's Hawks. WNV seroprevalence in our population was significantly correlated to the number of infected dead birds in the state during the previous year and may be the result of infections from the previous year, possibly during migration or winter.

The correlation of kestrel seroprevalence to the number of infected dead surveillance birds may result from kestrels being exposed to infected mosquitoes at a similar rate as other birds or from ingesting infected birds as part of their diet, as WNV can be transmitted by ingesting infected prey (Komar et al. 2003, Augsten et al. 2004). There were no significant correlations between WNV seroprevalence in kestrels and dead bird, mosquito, or human infections during the same year. This, together with the fact that WNV season in Pennsylvania begins after the kestrel breeding season ends, indicated that the American Kestrels nesting near Hawk Mountain are not good predictors of WNV infections in wildlife or humans in eastern Pennsylvania.

We found detectible levels of antibodies in a small proportion of the nestlings we sampled (Table 2). This suggests either that these birds were exposed to infection early in life, or more likely, that they received some immunity from the adult female. Passive transfer of immunity from adults to chicks has been demonstrated experimentally in owls (Hahn et al. 2006) and domestic chickens (Nemeth and Bowen 2007), as well as in naturally infected Rock Pigeons (*Columba livia*; Gibbs et al. 2005). Unfortunately, we were not able to establish a link between maternal immunity and antibodies in the nestlings in this study. Additional research is needed to determine whether adults can transfer immunity to nestlings.

The kestrel population in the northeastern United States has been declining for several decades, with a more precipitous decline coincident with the introduction of WNV into North America (Sullivan and Wood 2005). WNV infection in Pennsylvania in dead birds, mosquitoes, and humans was highest in 2003 and has since declined (Helwig 2005, Pennsylvania WNV Surveillance Program 2008). The seroprevalence data from our study in-

dicates that the exposure of our study population was highest in 2003–04 and has since declined. Hawk Mountain Sanctuary has records of the numbers of kestrels using its nest boxes and of the numbers of American Kestrels migrating past the sanctuary from 1992 until 2007 (Medica et al. 2007, Farmer et al. 2008). These data suggest that although the numbers are not yet equivalent to numbers prior to the introduction of WNV into the continental United States (an average of 88 pairs per year from 1992–98; Medica et al. 2007), the local kestrel population may be beginning to recover. Forty-eight pairs of kestrels used nest boxes in 2007, and 51 pairs used them in 2008 (K. Bildstein unpubl. data), which was an increase from the lowest count (during the study period) of 35 pairs in 2005. The kestrel population in this part of Pennsylvania is subject to a number of stressors, including loss of farmland habitat and increased predation by other raptors (Farmer et al. 2006). Although we cannot show conclusively that WNV was responsible for the recent decline in the numbers of kestrels nesting near Hawk Mountain, the decline began when WNV entered the continental United States in 1999, and the decreased prevalence of WNV in Pennsylvania in recent years coincides with a slight increase in kestrel population numbers.

ACKNOWLEDGMENTS

This study would not have been possible without the dedication and hard work of Amy Williams, Rachael Clauser, Allison Stock, Jill Koppenhaver, Bob and Sue Robertson, Brad Silfies, Andrea Solinski, Sherry Schaeffer, Mark Voydik, Leah Kraft, and Susan Geist, all of whom were essential to field and/or laboratory components of the study. The authors thank Stan Reynolds and James Lute from the Pennsylvania Department of Health for conducting the RT-PCR analysis and Amy Glazer of the New York State Animal Health Diagnostic Lab for running the PRNT analyses. We also thank Mike Sukhdeo, Bim Angst, Justin Malmberg, and two anonymous reviewers for their helpful comments on the manuscript. This study was funded by the Advisory Board of the Pennsylvania State University, Schuylkill Campus, the Pennsylvania State University, Capital College Office of Research and Graduate Studies, and the Acopian Center for Conservation Learning. This is Hawk Mountain Sanctuary Contribution to Conservation Science No. 179.

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Received 12 September 2008; accepted 7 July 2009
Associate Editor: John A. Smallwood