DOI: 10.7589/2016-07-169

Journal of Wildlife Diseases, 53(3), 2017, pp. 586–590

Wild Birds, a Source of Reticuloendotheliosis Virus Infection for the Endangered Attwater's Prairie-Chicken (*Tympanuchus cupido attwateri*)?

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ABSTRACT: Reticuloendotheliosis virus (REV) infects a wide range of avian species. Since 1998, when it was first reported in a captive flock of the endangered Attwater's Prairie-chicken (*Tympanuchus cupido attwateri*; APC), REV has plagued APC recovery efforts. While REV frequently occurs in captive bird flocks throughout the world, including commercial poultry, the reservoir for initial infection of flocks is poorly understood. From 2008–16, 412 blood samples and 216 liver samples collected from 32 species of birds on or near Attwater Prairie Chicken National Wildlife Refuge in Colorado County, Texas, US, and 89 blood samples obtained from a Texas game farm that provides thousands of Northern Bobwhites (*Colinus virginianus*) and Ring-necked Pheasants (*Phasianus colchicus*) for hunting throughout Texas, were tested for REV by real-time PCR. Of the 717 samples, one liver sample from a Savannah Sparrow (*Passerculus sandwichensis*) and three blood samples from game farm Ring-necked Pheasants tested positive for REV. These data, although limited, indicate a low prevalence of REV in birds sharing or in close proximity to APC habitat. More-extensive surveillance testing is warranted to determine the spatial and temporal dynamics of REV in wild bird populations and the relative role these birds may play as potential reservoirs for maintaining REV infections in both the wild and captive setting.

Key words: Attwater's Prairie-chicken, duplex real-time PCR, high-throughput testing, reticuloendotheliosis virus, REV.

Reticuloendotheliosis virus (REV) is a type-C retrovirus of the genus *Gammaretrovirus* within the family *Retroviridae* (International Committee on Taxonomy of Viruses 2016). The REV most commonly occurs in the avian orders Anseriformes and Galliformes, affecting a variety of avian species including chickens, turkeys, ducks, geese, quail, and prairie chickens (Drew et al. 1998; Nair 2013; Niewiadomska and Gifford 2013). Although infection seems to be widespread, the appearance of disease is uncommon in wild birds. In commercial poultry, infection has been most-frequently associated with REV-contaminated vaccines such as fowl poxvirus vaccines (Tadese and Reed 2003; Nair 2013). Both the remnants of the REV long terminal repeat (LTR) or near full-length REV provirus have been shown to be integrated into the genome of fowl poxvirus and have been implicated in increased virulence (Garcia et al. 2003; Tadese et al. 2008). Avian poxviruses are maintained in nature and affect a wide range of avian species (US Geological Survey 1999). Interestingly, many outbreaks of REV in bird populations, particularly prairie chickens, have coincided with avian poxvirus outbreaks (Drew et al. 1998; Barbosa et al. 2007; Niewiadomska and Gifford 2013).

In 1998, REV was first reported in captive prairie chicken flocks, including those of the endangered Attwater's Prairie-chicken (APC, *Tympanuchus cupido attwateri*; Drew et al. 1998; Zavala et al. 2006). While infection of APC with REV in captive flocks continues to plague recovery efforts, prevalence of REV in free-ranging APC and other galliforms is generally low, at 0–3% (Peterson et al. 2002a, b; Wiedenfeld et al. 2002; Jiang et al. 2013), although Ingram et al. (2013) found antibodies to REV in 15/24 (63%) Wild Turkeys (*Meleagris gallopavo*). While REV has been documented in numerous bird species, the natural reservoirs and epizootiology for this disease are poorly understood

(Witter and Johnson 1985; Barbosa et al. 2006; Nair et al. 2013). The virus can be transmitted both horizontally and vertically by direct contact with infected birds (Griffin 1998). Also, REV has been demonstrated to be transmitted horizontally by vectors, specifically mosquitos (*Culex pipiens*, *Culex quinquefasciatus*) and house flies (*Musca domestica*), in laboratory and experimental field conditions (Motha et al. 1984; Davidson and Braverman 2005). The objective of our study was to determine if birds that share habitat with wild or captive APC could serve as sources of REV.

From 2008–15, we collected samples in and around Attwater Prairie Chicken National Wildlife Refuge (Austin and Colorado counties, Texas, USA; 29°40′N, 96°16′W), which contains one of two remaining noncaptive APC populations. Blood samples (≥0.1 mL) were obtained by venipuncture, placed in a tube containing an anticoagulant (heparin or ethylenediaminetetraacetic acid), and frozen (–20 C) until tested. Most protected species were obtained by mist netting (permit 22280-D) on Attwater Prairie Chicken National Wildlife Refuge and were released onsite. House Sparrows (*Passer domesticus*) were also captured by mist netting near Sealy, Texas, in adjacent Austin County (29°44′N, 96°09′W). Northern Bobwhite (*Colinus virginianus*) liver samples were collected from hunters during hunting season in Colorado County, Texas (29°40′N, 96°16′W); livers were pooled (≤3/pool) and frozen (–20 C) until tested (25 samples representing 65 individuals). Waterfowl liver samples were obtained from hunter processing stations during hunting season Eagle Lake, Texas (29°36′N, 96°18′W). Unhatched APC embryos were collected from Goliad County, Texas (28°36′N, 97°24′W; permits SPR-0491-834 and TE051839). Brown-headed Cowbirds (*Molothrus ater*), grackles (*Quiscalus* spp.), and Red-winged Blackbirds (*Agelaius phoeniceus*) were collected by Texas Wildlife Services personnel (migratory bird permit 714649) in Colorado County, Texas (29°40′N, 96°16′W) and whole birds were frozen (–20 C) until liver samples were collected. In 2016, blood samples were collected from Northern Bobwhites (*Colinus virginianus*) and Ring-necked Pheasants (*Phasianus colchicus*) located on a game farm that provides birds for hunting throughout Texas. All samples were sent to the Texas A&M Veterinary Medical Diagnostic Laboratory for REV testing using real-time PCR methods as previously described (Sun et al. 2011). In brief, liver samples were thawed, homogenized in 1 mL phosphate buffered saline, briefly centrifuged to remove large particulates, and 50 µL of the supernatant was added to lysis binding buffer for nucleic acid extraction using a magnetic bead extraction method (MagMAX™ AM1836, Applied Biosystems/Thermo Fisher Scientific, Foster City, California, USA) and a magnetic particle processor (KingFisher 96, Thermo Fisher Scientific). Blood samples were thawed, mixed thoroughly, and then a 1-µL aliquot of the blood sample was added to lysis binding buffer for nucleic acid extraction. The real-time PCR assay is a primer/probe-based multiplex assay consisting of two viral targets, the LTR and the envelope gene (*env*) and an exogenous internal control which serves as an internal control for inhibition of the extraction and PCR reaction. The PCR assay was performed and analyzed using an AB 7500 Fast instrument (Applied Biosystems/Thermo Fisher Scientific); samples with a cycle threshold ≤36 for both targets (LTR and *env*) were considered positive; cycle threshold values were not reported by the laboratory.

A total of 717 samples (501 blood samples and 216 liver samples) were collected from birds representing 33 species (Table 1). Of the 628 samples tested from wild birds, only one (0.2%) liver collected from a Savannah Sparrow (*Passerculus sandwichensis*) tested positive for REV. This is the first report of an REV-positive passeriform in North America; although Jiang et al. (2013) reported positives from two Passeriformes families in China. Of the game farm birds, none of 64 blood samples from Northern Bobwhites tested positive, but three of 25 (12%) blood samples from Ring-necked Pheasants tested positive (Table 1).

TABLE 1. Results, by species, of reticuloendotheliosis virus multiplex real-time PCR testing of blood and liver samples collected from wild birds (*n*=628) in Austin and Colorado counties, Texas, USA, from 2008–15 and blood samples collected from Northern Bobwhites (*Colinus virginianus*; *n*=64) and Ring-necked Pheasants (*Phasianus colchicus*; *n*=25) at a Texas game farm in 2016.

Species	Scientific name	Blood		Liver	
		No. sampled	No. positive	No. sampled	No. positive
American Goldfinch	*Spinus tristis*	2	0	0	0
American Pipit	*Anthus rubescens*	1	0	0	0
Attwater's Prairie-chicken	*Tympanuchus cupido attwateri*	7	0	9[a]	0
Blue-winged Teal	*Anas discors*	0	0	15	0
Brown-headed Cowbird	*Molothrus ater*	0	0	8	0
Carolina Chickadee	*Poecile carolinensis*	3	0	0	0
Chipping Sparrow	*Spizella passerina*	7	0	0	0
Common Yellowthroat	*Geothlypis trichas*	1	0	0	0
Eastern Meadowlark	*Sturnella magna*	1	0	0	0
Eastern Phoebe	*Sayornis phoebe*	2	0	0	0
Grackle spp.	*Quiscalus spp.*	0	0	4	0
Greater White-fronted Goose	*Anser albifrons*	0	0	38	0
Green-winged Teal	*Anas crecca*	0	0	9	0
Harris' Sparrow	*Zonotrichia querula*	5	0	0	0
House Sparrow	*Passer domesticus*	27	0	1	0
Killdeer	*Charadrius vociferus*	2	0	1	0
Le Conte's Sparrow	*Ammodramus leconteii*	1	0	0	0
Lincoln's Sparrow	*Melospiza lincolnii*	2	0	0	0
Loggerhead Shrike	*Lanius ludovicianus*	2	0	0	0
Northern Bobwhite (wild)	*Colinus virginianus*	0	0	25[b]	0
Northern Bobwhite (game farm)	*C. virginianus*	64	0	0	0
Northern Pintail	*Anas acuta*	0	0	15	0
Northern Shoveler	*Anas clypeata*	0	0	14	0
Red-winged Blackbird	*Agelaius phoeniceus*	16	0	22	0
Ring-necked Pheasant (game farm)	*Phasianus colchicus*	25	3	0	0
Ross' Goose	*Chen rossii*	0	0	2	0
Sedge Wren	*Cistothorus platensis*	3	0	0	0
Song Sparrow	*Melospiza melodia*	2	0	0	0
Savannah Sparrow	*Passerculus sandwichensis*	307	0	10	1
Snow Goose	*Chen caerulescens*	0	0	3	0
Swamp Sparrow	*Melospiza georgiana*	2	0	0	0
Vesper Sparrow	*Pooecetes gramineus*	4	0	0	0
White-crowned Sparrow	*Zonotrichia leucophrys*	15	0	0	0
Total		501	3	176[c]	1

[a] Includes six unhatched embryos from two hens; two and four eggs/hen, respectively.

[b] Represents 65 individuals; livers were pooled (2 or 3 per pool) for testing.

[c] Represents 216 individuals; some samples were pooled for testing.

In places where avian species are concentrated, such as commercial poultry operations, REV is common (Nair et al. 2013). The detection of REV in three of 25 Ring-necked Pheasants from the game farm supports this observation. Ring-necked Pheasants may have a higher probability of exposure to REV due to their age and length of time cultivated prior to release (6–8 mo versus 3–4 mo for Northern Bobwhites). Similarly, captive flocks

of birds maintained for conservation purposes, such as the APC, are also susceptible (US Fish and Wildlife Service 2010). In contrast, REV in wild birds is typically at low prevalence, as found in this study. However, even at low prevalence wild birds may spread REV to new areas and provide a potential source of infection of captive flocks (Reed et al. 2003). The Gulf of Mexico coastal prairie has among the highest densities (>300/km^2) of breeding land birds in North America (Blancher et al. 2007) and hosts millions of overwintering waterfowl and migratory land birds representing 350–400 species (Smeins et al. 1992). The diversity and sheer number of migrating or overwintering birds throughout the coastal prairie region suggests the possibility for increased risk of disease transmission, including REV. Furthermore, risk may be higher today than historically because of the increased bird diversity which has occurred due to an increase in the mosaic of prairie, successional forested areas, and agriculture lands over the last century (Knopf 1994).

The relationship between poxvirus and the maintenance of REV in captive and free-ranging birds remains to be determined. Avian poxviruses are maintained in nature and mosquitos can serve as mechanical vectors of transmission; however, it is uncertain whether poxviruses in nature routinely carry full-length REV, serving as a source of infection, or a bird is infected with REV, causing immunosuppression and thereby increasing susceptibility to poxvirus infection. While the role of free-ranging birds in the etiology of REV as it pertains to captive flocks remains unclear, further investigation is merited in order to develop more-effective strategies for managing this disease as part of the ongoing efforts to maintain APC populations.

The authors wish to thank F. Sun, J. Meier, J. Khal, J. Swensen, D. Roach, T. J. Muir, R. Jurries, and K. Borgstrom for their expert technical and logistical assistance. This work was a collaborative effort between the Texas A&M Veterinary Medical Diagnostic Laboratory, Texas A&M University College of Veterinary Medicine, US Fish and Wildlife Service Attwater Prairie Chicken National Wildlife Refuge, Houston Zoo, Inc., and Texas Parks and Wildlife Department. The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the US Fish and Wildlife Service or any of the aforementioned organizations.

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Submitted for publication 20 July 2016.
Accepted 16 December 2016.