The Prevalence of West Nile Virus Antibodies in Blood Samples from Song Birds Collected from the Fountain Creek Region of Colorado

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ABSTRACT

West Nile Virus (WNV) is a positive strand RNA virus (*Flaviviridae*) that is transmitted by mosquitoes (Culex species). The virus is normally maintained and amplified in avian reservoir hosts, but infected mosquitoes also bite humans, horses, and other vertebrates which can result in the transmission of the virus. WNV infections have been reported all over North America, including recent infections in Colorado. Mosquitoes are routinely sampled for the presence of WNV, but bird populations are more difficult to trap and analyze. In a collaborative effort, birds were trapped, banded, and blood samples were collected in the Fountain Creek Region of Colorado in summers of 2014–16. Blood samples were screened for WNV antibodies using an indirect ELISA (enzyme-linked immunosorbent assay). Initial screening results have detected birds being positive for WNV antibodies. We compared WNV prevalence between families of birds. It was predicted that *Icteridae*, the blackbird family, would show higher WNV antibody presence than other avian families because previous studies have shown that Common grackles (*Quiscalus quiscula*), a member of the *Icteridae* family, have higher amounts of viremia. We have found that WNV antibodies were more present in Common grackles (*Q. quiscula*) than all other songbirds caught.

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INTRODUCTION

West Nile Virus (WNV) was first observed in the West Nile district of Uganda in 1937 from the *Culex pipiens* mosquito (Henning et al., 2015). It was later found in Asia, the Middle East, and Europe with additional Culex species (Asnis et al., 2000). WNV was first detected in the U.S. in 1999 (Henning et al., 2015). Since its introduction in the U.S., WNV has infected over 1.5 million people which has resulted in substantial disease in over 300,000 people (Henning et al., 2015). WNV has also lead to nearly 13,000 cases of encephalitis and meningitis, often leading to death (Kilpatrick, 2011). Although WNV has been studied a lot since its introduction to the United States in 1999, there are still no vaccines or medications to treat the virus (Davis et al., 2001). WNV has been detrimental to many vertebrate species including birds, humans, and horses (Komar, 2000). WNV has since been maintained in an enzootic cycle involving ornithophilic (bird biting) mosquitos as transmission vectors and birds as reservoir hosts (Pérez-Ramírez et al., 2014). WNV has had a severe impact on the bird populations all around North America. The American Crow population was steadily thriving in 1998, but since the introduction of WNV in 1999, there has been a rapid decrease in their population (LaDeau et al., 2007). Specifically, the American Crow population declined 45% since the arrival of WNV to the United States (LaDeau et al., 2007).

Birds have long been used as relevant model organisms for arbovirus studies as well as surveillance and serologic surveys because birds serve as primary reservoirs for many viruses including WNV, St. Louis encephalitis virus (SLEV), and Western equine encephalomyelitis virus (WEEV) (Tsiodras et al., 2008). To screen birds for the presence of WNV, serological tests such as an indirect enzyme-linked immunosorbent assay (ELISA) give accurate results in a short time period. An Indirect ELISA was originally used to detect seroreactivity against SLEV and WEEV but has now been modified for detecting anti-WNV antibodies in wild avian species. Two-step ELISAs involve two different binding steps with a primary antibody and a labeled secondary antibody. Indirect ELISAs use a secondary antibody that is conjugated with a reporter that can easily be detected in a high throughput assay. Advantages for using an Indirect ELISA include high sensitivity, flexibility, low cost, and lower levels of biocontainment since there is no intentional use of infectious virus, such as in PRNT assays. However, infectious virus could still be present in the blood samples, therefore, proper biosafety protocols should be followed when working with blood. Alternatively, active viral infections have been detected in bird blood samples using qRT-PCR to detect the viral RNA, or a sandwich ELISA to detect the viral antigen. However, those methods require the bird to be viremic, meaning that the virus is present in the bloodstream at the time of sampling, which is a narrow period of time and can be difficult to detect (Jozan et al., 2003). Seroprevalence is an easier approach to detect the overall burden of WNV on a bird population because the stability and detection of WNV antibodies is longer and more robust (VanDalen et al., 2013).

Mosquito feeding patterns modulate the encounter rates between the avian host and potentially WNV infected mosquitoes (Medeiros et al., 2016). Since different mosquito species have been shown to feed on different bird families, it might help us understand why certain families are showing higher WNV antibody presence than others (Medeiros et al. 2016). It appears that the *Culex pipiens* mosquito prefer human as well as avian hosts rather than others such as am-

phibians (Victoriano Llopis et al. 2016). In previous studies, several bird species have shown higher WNV titers including American Robins (*Turdus migratorius*), American Crows (*Corvus brachyrhynchos*), and Common Ravens (*Corvus corax*) (Koenig et al. 2010). These species belong to the order Passeriformes and show higher WNV antibody presence than other avian species. House Sparrows (*Passer domesticus*) are an interesting species to note because of their new resistance to the NY99 WNV strain (Duggal et al., 2014). House Sparrows were a highly competent host for WNV, however, their competence has drastically decreased over time due to them developing resistance to the NY99 strain (Duggal et al., 2014). In order for a virus to replicate, there must be a certain level of host competence, meaning the host must be able to transfer the virus to another susceptible host, and this level has been found almost exclusively in birds. Birds not only transfer the virus, but they also act as biological reservoirs that amplify the virus. The species that belong to the order Passeriformes, as well as others, have been shown to have higher WNV presence because of their ability to allow the virus to replicate successfully, which is a characteristic of host species in specific host-virus-vector system (Pérez-Ramírez et al., 2014). The Fountain Creek areas in Southern Colorado are home to a wide variety of song birds and provide a great opportunity to sample the wild bird population for West Nile Virus seroprevalence. Pueblo County and other Southern Colorado areas have had consistent West Nile Virus cases since the early 2000's (Park et al., 2015).

The human population in southern Colorado continues to grow and a better understanding of the West Nile Virus burden on the local avian reservoirs can lead to a more complete picture of West Nile Virus prevalence and risks. We hypothesized that we would find significant levels of West Nile Virus antibodies in the wild song birds in the Fountain Creek region. We also expected that the viral burdens would be highly dependent on the species of bird samples. It was predicted that *Icteridae*, the blackbird family, would show higher WNV antibody presence than other avian families because previous studies have shown that Common grackles (*Quiscalus quiscula*), a member of the *Icteridae* family, have higher amounts of viremia. It was also predicted that House Sparrows (*P. domesticus*), member of the Passeridae family, would show low WNV antibody presence due to their increase in resistance.

MATERIALS AND METHODS

Sample Collection

Birds were caught using mist nets and playback of common bird species from 12 different sites in Southern Colorado within 0.25 miles of Fountain Creek in the summers of 2014–2016. Song birds are collected in the summer because it is safer for the birds. The nets were either six meter, nine meter, or twelve meters in length and were setup along the trees. MP3 players were used to attract the birds to the net. All nets were checked every 30 minutes. The birds were tagged with United States Fish and Wildlife Services (USFWS) aluminum bands, identified to species, and age and sex were recorded. A blood sample was collected from the brachial vein of each bird using a 27G needle. Approximately 100uL of blood was collected in heparinized microcapillary tubes. Birds were released unharmed at the site of capture. Blood samples were returned to the lab and stored at -20 °C until analysis.

ELISA Assay

Blood samples were diluted (1:100) in Phosphate-buffered saline (PBS) with 0.05% Tween and 0.5% Bovine Serum Albumin (BSA) and stored in a -20°C freezer for later use. Wells from an Immulon 2HB-High Binding 96 well plate were coated with 0.50ug/well, 500ng, of diluted antigen (1:1 Recombinant WNV preM peptide and WNV e protein) (1:100 with coating buffer, 0.015M Na_2CO_3 and 0.035M $NaHCO_3$). The antigen was placed in the first and second rows while only the coating buffer was placed in the third row. The plate was incubated at 37°C overnight to allow the antigen to attach to the bottom of the plate. Any unbound antigen was removed from the wells by washing 3 times with PBS with 0.05% Tween for five minutes per wash. Blocking solution (PBS with 0.05% Tween and 2.0% Casein) was placed inside the wells and left to incubate at 37°C for one hour. The blocking solution was then removed and 50uL of diluted whole blood was placed in designated wells (3 wells per sample). After the plate was incubated at 37°C for one hour, the diluted blood sample was removed from the wells and disposed of in bleach. The wells were washed 3x with PBS with 0.05% Tween for five minutes per wash. After washing, 50uL diluted goat anti-wild bird immunoglobulin peroxidase conjugated antibody (diluted 1:1000 in PBS with 0.05% Tween and 0.5% BSA) was placed in each well and incubated for one hour at 37°C. The plate was washed 3x with PBS with 0.05% Tween for five minutes each wash. Then 50uL of tetramethylbenzidine (TMB) peroxidase substrate (ratio 1:1) was added to each well and left to sit for five minutes. The reaction was stopped with the addition of 50uL of 2M HCl to each well. The results were detected and recorded using a BioRad plate reader detecting absorbance at 450nm. Positive/Negative (P/N) values were calculated for each bird caught by taking the average absorbance readings from the wells that contained WNV antigen and dividing by the well with only coating buffer. These values were calculated for each bird sample. The three values that the birds were classified in were negative for WNV antibodies ($P/N < 2.0$), marginally positive ($2 < P/N < 3$), and definitively positive ($P/N > 3$) (Ebel et al. 2002). Any individuals with $P/N > 2$ was considered to be positive for WNV for the statistical analysis in this study.

Statistical Analysis

We compared the P/N values and the proportion of infected individuals between families of birds. Families with fewer than 10 individuals captured were excluded from the analysis. These values were calculated by taking the average of the two wells that contained antigen and dividing that value by the well that contained only coating buffer. P/N values were compared using a general linear model with family as a fixed effect. Proportion of infected individuals was analyzed using a generalized linear model with a binomial distribution and a logit link function and family as a fixed effect. Posthoc comparisons between families included a sequential Bonferroni adjustment for multiple comparisons. Statistics were performed in SPSS 19 (IBM) with significance at $p = 0.05$.

RESULTS

A total of 483 individuals, 61 species in 20 families (Table 1), were screened for WNV antibodies using an Indirect ELISA. Half of the families were excluded from the statistical analysis due to small sample size ($n<10$). The average P/N value differs between families ($F = 2.77$, $df = 9, 375$, P

< 0.01, Fig. 1). The Icteridae showed significantly higher average P/N values than Parulidae (P < 0.01) and Passeridae (P < 0.01, Fig 1). Family also effected the proportion of infected individuals (Wald $\chi 2 = 16.88$, df = 9, p = 0.05, Fig 2) with Icteridae having a higher proportion of infected individuals than Parulidae (p = 0.05) and Passeridae (p < 0.001).

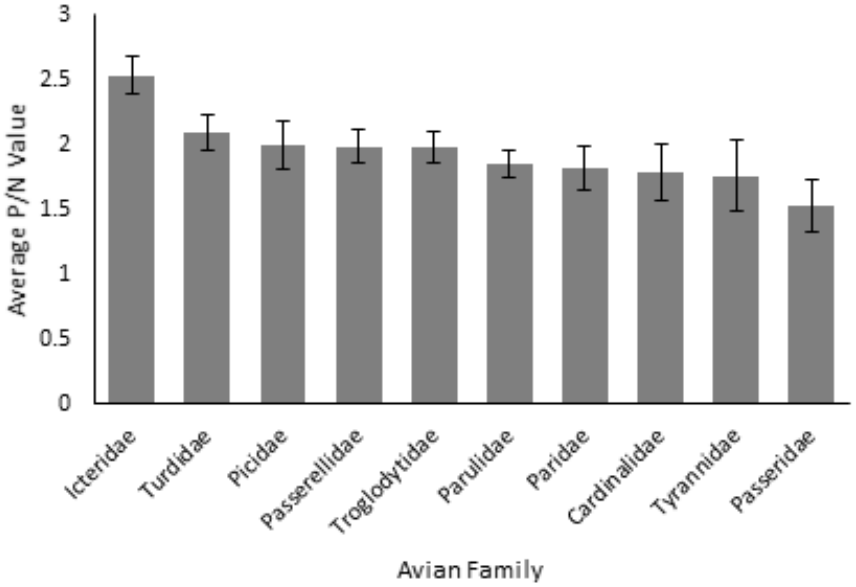

Figure 1: Average P/N value for each family of birds captured (n>10). Error bars represent one standard error.

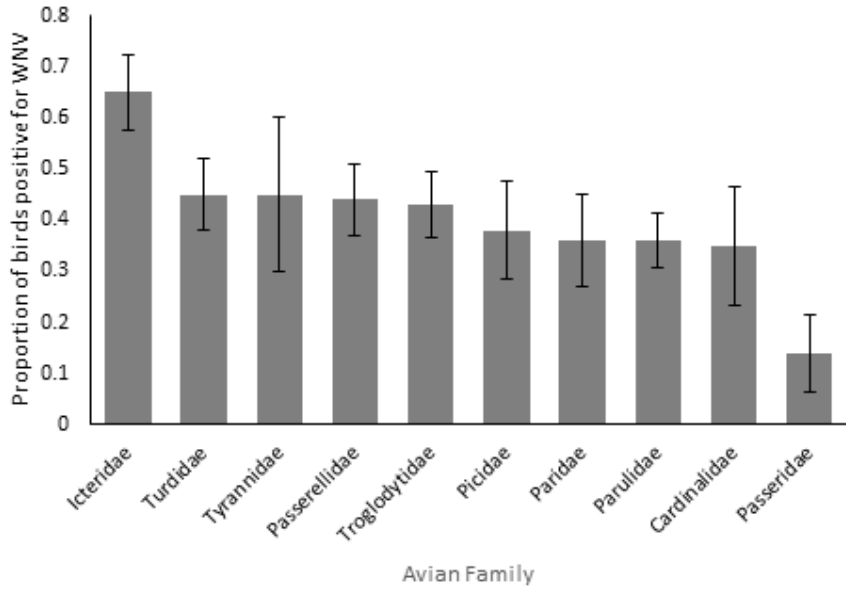

Figure 2: Proportion of birds in each family that tested positive for WNV (n>10). Bars represent one standard error.

Family	Scientific Name	Common Name	Sample Size	# of Birds with P/N>2
Aegithalidae	*Psaltriparus minimus*	Bushtit	1	0
Cardinalidae	*Pheucticus melanocephalus*	Black-headed Grosbeak	8	3
	Passerina caerulea	Blue Grosbeak	2	0
	Passerina amoena	Lazuli Bunting	5	3
	Piranga ludoviciana	Western Tanager	2	0
Corvidae	*Cyanocitta cristata*	Blue Jay	2	1
	Aphelocoma californica	Western Scrub-Jay	1	1
Emberizidae	*Zonotrichia leucophrys*	White-crowned Sparrow	1	0
Fringilidae	*Carduelis tristis*	American Goldfinch	2	0
	Carduelis psaltria	Lesser Goldfinch	2	0
	Spinus pinus	Pine siskin	4	2
Hirundinidae	*Hirundo rustica*	Barn Swallow	1	0
Icteridae	*Molothrus ater*	Brown-headed Cowbird	3	0
	Icterus bullockii	Bullocks Oriole	6	3
	Quiscalus quiscula	Common Grackle	26	18
	Agelaius phoeniceus	Red-winged Blackbird	8	7
Mimidae	*Toxostoma rufum*	Brown Thrasher	2	0
	Dumetella carolinensis	Gray Catbird	3	1
Paridae	*Poecile atricapillus*	Black-capped Chickadee	26	10
	Poecile gambeli	Mountain Chickadee	2	0
Parulidae	*Vermivora virginiae*	Yellow-rumped Warbler	12	3
	Vermivora pinus	Blue-winged Warbler	1	1
	Geothlypis trichas	Common Yellowthroat	8	4
	Oporornis tolmiei	McGillivray's Warblers	7	1
	Dendroica coronata	Myrtle Warbler	2	1
	Vermivora celata	Orange-crowned Warbler	1	0
	Vermivora peregrina	Tennessee Warbler	1	1
	Vermivora virginiae	Virginia's Warbler	2	1
	Wilsonia pusilla	Wilson's Warbler	2	1
	Icteria virens	Yellow-breasted Chat	13	3
	Setophaga petechia	Yellow Warbler	33	14
Passerellidae	*Spizella passerina*	Chipping Sparrow	6	2
	Junco hyemalis	Gray-headed Junco	3	2
	Melospiza lincolnii	Lincoln's Sparrow	3	1
	Junco hyemalis	Orange Junco	1	1
	Melospiza melodia	Song Sparrow	22	7
	Pipilo maculatus	Spotted Towhee	12	9
Passeridae	*Passer domesticus*	House Sparrow	20	3

Picidae	*Picoides pubescens*	Downy Woodpecker	10	4
	Sphyrapicus nuchalis	Red-naped Sapsucker	3	0
	Colaptes auratus	Red Shafted Flicker	13	6
Sittidae	*Sitta pygmaea*	Pygmy Nuthatch	3	0
	Sitta carolinensis	White-breasted Nuthatch	3	1
Regulidae	*Regulus calendula*	Ruby-crowned Kinglet	1	0
Sturnidae	*Sturnus vulgaris*	European Starling	1	1
Troglodytidae	*Thryomanes bewickii*	Bewick's Wren	5	2
	Troglodytes aedon	House Wren	49	22
Turdidae	*Turdus migratorius*	American Robin	46	22
	Catharus guttatus	Hermit Thrush	1	0
	Catharus ustulatus	Swanson's Thrush	1	0
Tyrannidae	*Empidonax occidentalis*	Cordilleran Flycatcher	3	2
	Empidonax oberholseri	Dusky Flycatcher	1	1
	Empidonax hammondii	Hammond's Flycatcher	2	0
	Empidonax minimus	Trail's Flycatcher	5	2
Vireonidae	*Vireo plumbeus*	Plumbeous Vireo	1	1
	Vireo olivaceus	Red-eyed Vireo	1	0
	Vireo gilvus	Warbling Vireo	6	1

Table 1: Names, sample size, and amount of birds that had P/N values > 2. The families that contained less than ten birds were excluded from the statistical analysis but are still shown in this table.

DISCUSSION

There were 20 different avian families sampled and half of them were included in the statistical analysis because they had at least ten birds in the sample. *Icteridae* had the highest average P/N value and mean infection value out of all other families sampled. *Icteridae* had a higher probability of infection than *Parulidae*. Although other families did not show significantly different values, there are still trends that can be seen (Figure 1 and Figure 2).

The *Passeridae*, which only includes *Passer domesticus* (house sparrows), showed the lowest P/N average as well as the lowest proportion of infected individuals. House sparrows have been suggested to serve as important reservoir hosts for WNV (Reisen et al. 2005). However, it has recently been detected that house sparrows are developing increased resistance to certain WNV strains (Duggal et al. 2014). The decrease in WNV seroprevalence that we have detected in sparrows in Southern Colorado may be due to this increased resistance. Additionally, house sparrows are invasive species that came to the United States from Europe and perhaps their European ancestry has helped with an underlying level of resistance to West Nile virus or similar viruses. Since WNV was present in Europe before coming to the United States, it is possible that house sparrows had exposure before invading the United States in the late 1800's (Roehrig 2013).

Although *Corvidae* were screened, they were not included in the analysis because only three birds in this family were captured. However, of the three birds, two showed high P/N values meaning

that they had WNV antibodies detected. There were two species caught in this set; the Blue Jay (*Cyanocitta cristata*) and Western Scrub Jay (*Aphelocoma californica*). In a previous study done in Texas, 11 out of 30 Blue Jays tested positive for WNV (Wilkerson et al., n.d.) Other studies have also observed higher WNV antibody presence in other members of the Corvidae, American crows (*C. brachyrhynchos*), and common ravens (*Corvus corax*) (Caffrey et al., 2005) (Yaremych et al., 2004) (Lindsay et al., 2003). There have also been many studied that have discovered high mortality rates among American Crows (*C. brachyrhynchos*) and Blue Jays (*C. cristata*) (McLean et al., 2001). It has also been demonstrated that seropositivity and mortality varied in avian response to WNV across order, family, and species (Verdugo et al., 2016).

It is important to note that Indirect ELISAs do not detect active virus. The purpose of an Indirect ELISA is to detect antibodies to the selected pathogen. In order to confirm viremia in the bird samples, qRTPCR or a direct ELISA would need to be performed (Jozan et al., 2003). An active virus measured in samples is hard to detect due to the short amount of time that birds are viremic. For example, experimentally infected American Robins (*Turdus migratorius*) with various doses of WNV only showed detectable levels of viremia between 3-7 days post injection. After the seventh day, there were no viremia titers detected in the birds (VanDalen et al., 2013). Detecting infectious virus in wild-caught birds is rare and we chose to look at antibody prevalence to understand infection levels at a more population-based level.

This study suggests that different avian families have different amounts of exposure to WNV. It is uncertain as to why some families showed significance while others did not, however this study is valid because some families show significance and there are trends that are followed throughout the dataset. Our findings have also been supported to other WNV studies on birds. Future studies will focus on determining if there is a significance in WNV antibodies in avian order, guild, age, and geographical location.

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